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**Effects of neutrophils and reactive nitrogen oxide
species in tumors as determined by genetic manipulation
of the *Mutatest* mouse tumor model**

Arsalan S. Haqqani

**Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in
partial fulfillment of the requirements for the degree of Doctor of Philosophy**

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Thesis Abstract

Most human tumors exhibit accumulation of mutations throughout their progression. In addition, many human tumors are infiltrated with inflammatory cells, such as neutrophils and macrophages. These cells can generate potentially mutagenic species, including nitric oxide-derived species. We hypothesize that these species contribute to the accumulation of mutations in tumors. Our lab previously developed the 'Mutatect' mouse tumor model to study this hypothesis. Mutatect fibrosarcoma cells can form solid tumors in C57BL/6 mice when injected subcutaneously. These tumors are variably infiltrated with neutrophils. The neutrophils contain inducible nitric oxide synthase, and tumor cells contain nitrotyrosine, an indicator of nitric oxide-derived damage. Neutrophil number correlates with mutation arising *in vivo* in Mutatect cells at the hypoxanthine phosphoribosyltransferase (*hprt*) locus, a marker of genetic damage. Dietary vitamin E significantly lowers the *hprt* mutations. I genetically engineered Mutatect cells to produce interleukin-8, a neutrophil attractant. Cells expressing high levels of interleukin-8 formed small tumors, whereas cells expressing lower levels produced large tumors. A biochemical assay was developed to quantify neutrophil content in tumors. Interleukin-8-expressing tumors had a significantly higher neutrophil content and *hprt* mutations than non-expressing tumors. Interleukin-8 gene instability was observed in high interleukin-8-expressing tumors. Dietary vitamin E dramatically inhibited both *hprt* mutations and interleukin-8 instability. It also affected neutrophil distribution in tumors. High level of protein nitrotyrosine was seen in tumors with high neutrophil content (and mutations). Using proteomics tools, a significant amount of nitrotyrosine could be identified in histones, the major nuclear proteins associated with DNA. Mechanism of nitric oxide-derived mutagenicity was also examined. Previous studies implicate the role of intracellular S-nitrosoglutathione, which was recently found to be detoxified by formaldehyde dehydrogenase. I developed several cell-lines with down-regulated formaldehyde dehydrogenase levels using RNA interference and antisense techniques. A metabolic cycle, involving formaldehyde dehydrogenase, capable of regulating protein S-nitrosation was identified. This thesis has contributed significantly towards understanding the role of inflammatory cells and the nitric oxide-derived factors in solid tumors. It has also allowed us to understand the mechanism(s) of S-nitrosation and tyrosine nitration in proteins, both of which may have physiological and pathological relevance.

To my parents

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Abbreviations

6-TG	6-thioguanine
8-nitro-dG	8-nitro-2'-deoxyguanosine
8-oxo-dG	8-hydroxy-2'-deoxyguanosine
ATA	3-amino-1,2,4-triazole
bp	basepair
BSA	bovine serum albumin
BSO	L-buthionine-(<i>S,R</i>)-sulfoximine
cDNA	complementary DNA
CDTA	2-diaminocyclohexane-N, N, N', N'-tetraacetic acid
CETAB	Hexadecyltrimethylammonium bromide
cGMP	cyclic guanosine monophosphate
CO ₂	carbon dioxide
CYSH	cysteine (reduced)
CYSNO	<i>S</i> -nitrosocysteine
DHEA	dehydroepiandrosterone
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DTPA	diethylenetriaminepentaacetic acid
ELISA	enzyme linked immunosorbant assay
eNOS	endothelial NOS
FDH	formaldehyde dehydrogenase
γ-GCS	γ-gultamylcysteine synthetase
GPx	glutathione peroxidase
GR	glutathione reductase
GS	glutathione synthase
GSH	glutathione
GSNO	<i>S</i> -nitrosoglutathione
GSSG	glutathione disulfide
GSSR	mixed disulfide with glutathione
GTN	glyceryl trinitrate (nitroglycerin)

H₂O₂	hydrogen peroxide
HOBr	hypobromous acid
HOCl	hypochlorous acid
HPLC	high performance LC
<i>hprt</i>	hypoxanthine phosphoribosyltransferase
IL-8	interleukin-8
iNOS	inducible NOS
IU	international units
kb	kilo-basepair
LC	liquid chromatography
MALDI	matrix-assisted laser desorption ionization
MS	mass spectrometry
MS/MS	tandem MS
MW	molecular weight
N₂O	dinitrogen monoxide
N₂O₃	dinitrogen trioxide (nitrous anhydride)
Na₂S₂O₄	sodium dithionite (sodium hydrosulfite, sodium sulfoxylate)
NAC	<i>N</i>-acetylcysteine
NAD⁺	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
NADP⁺	nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NCBI	national centre for biotechnology information
nESI	nanoelectrospray ionization
nNOS	neuronal NOS
NO_x	nitrogen oxides
NO⁻	nitrosonium anion
NO⁺	nitrosonium cation
NO[•]	nitric oxide (nitrogen monoxide)
NO₂⁻	nitrite
NO₂[•]	nitrogen dioxide

NO₂Cl	nitryl chloride
NOS	nitric oxide synthase
¹O₂	singlet oxygen
O₂⁻	superoxide
O₂	oxygen
OH[•]	hydroxyl radical
ONOO⁻	peroxynitrite
OPT	<i>o</i>-phthalaldehyde
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pro-SNO	<i>S</i>-nitrosated proteins
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
ROS	reactive oxygen species
ROS/NO_x	reactive oxygen and nitrogen species
RSH	sulfhydryl-containing compounds
RSNO	<i>S</i>-nitrosothiols
RSNO₂	<i>S</i>-nitrothiols
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of mean
siRNA	short-interfering RNA
SNAC	<i>S</i>-nitroso-<i>N</i>-acetylcysteine
SNP	sodium nitroprusside
SOD	superoxide dismutase
TBST	Tris-buffered saline with tween
TOFMS	time-of-flight MS
TRE	tTA response element
tTA	tetracycline-responsive transcriptional activator
<i>V</i>	volume

General Introduction

a) Background

i) Genetic instability in human cancers

The majority of human cancers (including solid tumors, leukemias, and lymphomas) are characterized by the accumulation of mutations, referred to as genetic instability (1). Mutations can be categorized into 2 major types: i) *chromosomal* abnormalities, which include gain or loss of chromosome (aneuploidy), chromosomal rearrangements resulting from DNA strand breaks (translocations, inversions, or other rearrangements), and gain or loss of portions of chromosomes (amplification, large-scale deletions); ii) *nucleotide sequence* abnormalities, which include point mutations (missense, nonsense) and small insertions or deletions (some of which result in frame-shift mutations). Mutations can occur throughout the genome but they contribute to cancer by affecting 2 major classes of genes: cellular proto-oncogenes and tumor-suppressor genes. Cellular proto-oncogenes normally function in cell-cycle progression and cellular proliferation. Mutational activation of a number of cellular proto-oncogenes has been observed in many human cancers (Table I.1). Mutations in proto-oncogenes result in the synthesis of an oncogenic protein product that can promote unregulated cell-cycle progression and cellular proliferation, hallmarks of cancerous cells. Tumor-suppressor genes, by contrast, are negative regulators of cellular proliferation and cell-cycle progression. Mutational *inactivation* of this class of genes is also observed in many human cancers (Table I.2). Functional or physical loss of tumor-suppressor genes *free*s the cell from the growth constraints imposed by their protein products, contributing to cancer development. Thus, the accumulation of mutations in both cellular proto-oncogene and tumor-suppressor genes, observed in many human cancers, can contribute to the process of tumor progression through the uncoupling of the normal controls that hold the cell cycle in check or promote cell-death.

ii) Mutations and cancer progression

During the last 50 years, the sequence of events comprising cancer development has been systematically dissected and the mechanism increasingly refined. Figure I.1 illustrates the widely accepted paradigm of cancer initiation and progression. The conversion of a normal cell into a malignant and a metastatic one requires multiple steps. Each step is characterized by a mutational event that confers some form of selective advantage for cell growth to the affected cell (1). Mutations that lead to activation of cellular proto-oncogenes

TABLE 1.1: Examples of mutated proto-oncogenes in human cancers.

Mutations		Tumor type
Proto-oncogenes	Type	
<i>c-ras</i>	Point-mutation	Thyroid, GI tract, uterus, lung, myelodysplastic syndromes, leukemias, pancreas, bile duct
	Amplification	Bladder, breast, lung and head and neck carcinomas
<i>c-myc</i>	Amplification	Leukemias, breast, stomach, lung, and colon carcinomas, neuroblastomas and glioblastomas
	Translocation	Burkitt's lymphoma and other hematopoietic tumors
<i>c-erbB</i>	Amplification	Glioblastomas, medulloblastomas and renal cell, squamous cell, breast, gastric, esophageal, salivary gland, lung, colon, and ovarian carcinomas

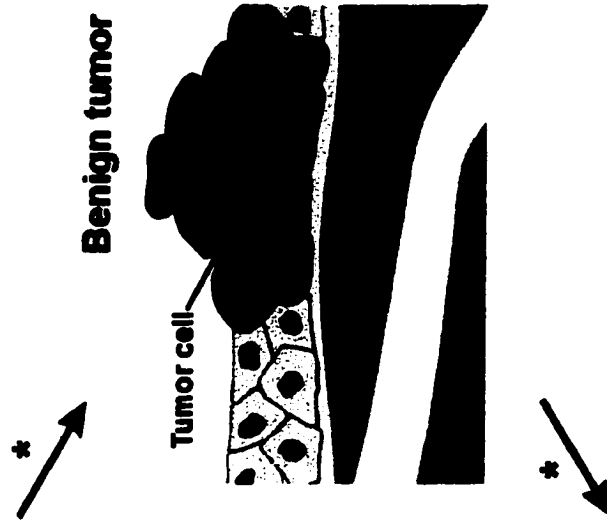
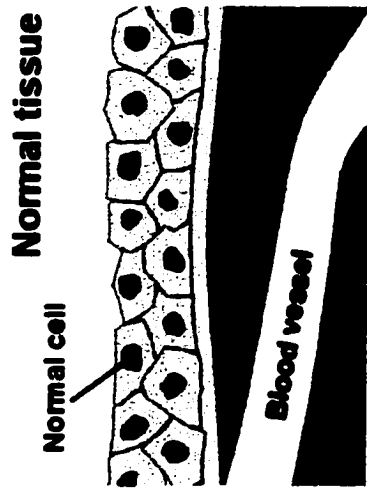
From ref. (2).

TABLE 1.2: Examples of tumor suppressor genes.

Tumor suppressor genes	Tumor types
<i>Rb1</i>	Retinoblastoma, sarcomas, bladder, breast, esophageal, and lung carcinomas
<i>p53</i>	Li-Fraumeni cancer family syndrome, bladder, breast, colorectal, esophageal, liver, lung, and ovarian carcinomas, brain tumors, sarcomas, lymphomas, and leukemias
<i>APC</i>	Familial adenomatous polyposis, colorectal, stomach, and pancreatic carcinomas
<i>BRCA1/2</i>	Hereditary breast cancer
<i>p16^{INK4A}</i>	Familial melanoma
From ref. (2)	

FIGURE 1.1. Schematic representation of the widely accepted model of cancer initiation and progression.

Each step in the development is characterized by a mutational event, such as activation of cellular proto-oncogenes or inactivation of tumor-suppressor genes. See text for more details



or inactivation of tumor-suppressor genes can provide such a selective advantage. The first mutational event in a single cell permits limited expansion of the progeny of that cell. Subsequent mutational events give rise to new clonal outgrowths with greater proliferative potential. Even after a malignant tumor is established, mutations continue to accumulate and further promote proliferative capacity and invasiveness of the tumor (1, 3). Thus, the initiation and progression of cancer is a multi-step process involving the accumulation of a succession of genetic changes. The accumulation of these changes is believed to be the driving force in the evolution of cancers.

iii) Factors contributing to mutational events in tumors

There are many factors that can contribute to the accumulation of mutations observed during cancer development. These may be divided into 2 types: environmental and biological factors. Environmental factors are usually extrinsic to human body and mainly involve the chemical carcinogens many humans are exposed to, inhale or ingest (e.g., asbestos, tobacco smoke, arsenic, chromium, etc.). Based upon variations in populations, approximately 50% of human cancer deaths have been shown to be associated with environmental agents (predominantly cigarette smoking) (4).

Biological factors are usually intrinsic to the human body. These may be further divided into 2 subtypes: *internal* and *external* factors. Internal factors refer to those mechanisms of genetic instability that are intrinsic to the tumor cell itself (e.g., mutator phenotype) or to a “normal” cell carrying a gene that predisposes to the development of cancer. Some investigators have found the mutation frequency in malignant cells is significantly higher than that of corresponding normal cells (1, 4). A prime example is the “mutator phenotype” postulated by Loeb’s group (4). According to this model, a mutation in a “mutator gene” (caused by environmental or other biological factors) early in the development of cancer is responsible for the accumulation of subsequent mutations. Mutator genes may include genes encoding DNA repair enzymes and genes affecting cell-cycle check-points (e.g., p53), since mutations in these genes can predispose cells to accumulation of further mutations. As a consequence of these mutations, an enhanced mutation frequency is observed in some cancer cells compared to normal cells (4). However, mutations in mutator genes do not always enhance the mutation frequency in tumor cells. Nor do all tumor cells exhibit a high intrinsic mutation frequency compared to normal cells (1). This

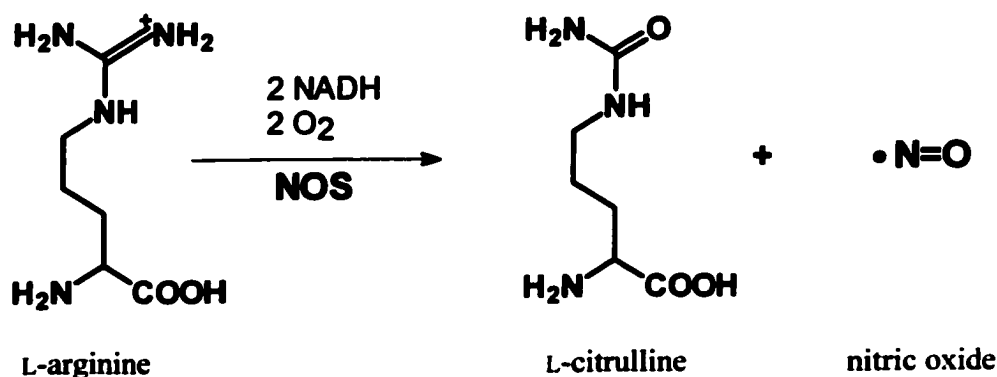
suggests that the internal factors, on their own, may not explain the accumulation of mutations in cancer development.

“External factors” are those mutagenic factors produced within our bodies that are extrinsic to a tumor cell or to a normal cell destined to become a tumor cell. Although they are not as well studied as internal factors, evidence for the involvement of external factors in cancer development is building. Most prominent among these factors are i) reactive oxygen species (ROS) and ii) nitric oxide (NO[•])-derived species. These species are released into the extracellular microenvironments of tissues and tumors by various cell types, especially in high amounts by inflammatory cells such as macrophages and neutrophils (see below). Many types of ROS are capable of causing DNA damage and hence are potentially mutagenic. As much as 1-4% of the oxygen (O₂) metabolized by the body can form ROS (5). Superoxide (O₂^{•-}), one of the major ROS, is synthesized by specific enzymes such as NADPH oxidase or xanthine oxidase (6) and under certain conditions by nitric oxide synthases (7-9), but also occurs as unwanted by-products of normal metabolism (6). It can spontaneously or enzymatically (by superoxide dismutase (SOD)) be converted into hydrogen peroxide (H₂O₂), which itself is a potentially damaging molecule and can form the highly DNA-damaging hydroxyl radical (OH[•]) (5). H₂O₂ is also used by the myeloperoxidase-chloride system in inflammatory cells to release potentially DNA- and protein-damaging hypochlorous acid (HOCl) (6). HOCl may further react with H₂O₂ to produce another ROS, singlet oxygen (¹O₂) (10) that is capable of inducing direct DNA damage (5). NO[•] is another external factor that is also reactive and damaging. However, *when NO[•] reacts with molecular oxygen or ROS, it generates various types of reactive nitrogen oxide species (NO_x) that are significantly more reactive and damaging than ROS or NO[•] themselves (see below) (5, 11-13).* Over the last decade, these NO_x species have emerged as key factors in the pathology of many inflammatory disorders, including cancer. Both ROS and NO_x have been shown to possess many characteristics of carcinogens. Mutagenesis caused by these species is believed to contribute to the *initiation* of cancer (13); however, the mutagenic role of ROS/NO_x in the later phases of cancer has not been well-studied.

iv) *NO[•]-derived reactive NO_x species*

Nitric oxide (NO[•]) is a potent biological mediator that has triggered an explosion of scientific studies over the last decade. Research in this field has identified diverse physiological and patho-physiological roles for NO[•] as a vasodilator, neurotransmitter, antimicrobial effector molecule and immunomodulator. Following its identification as an *endothelium-derived relaxing factor*, NO[•] has been shown to be produced by such cell types such as macrophages, neutrophils, endothelial cells, neurons, hepatocytes, fibroblasts, epithelial cells, smooth muscle cells, cardiac myocytes and many more.

Biosynthesis of NO[•] is a result of five-electron oxidation of one of the chemically equivalent guanidine nitrogens of L-arginine through the action of nitric oxide synthases (NOS), forming the free radical NO[•] and a by-product L-citrulline (14).



Three distinct isoforms of the NOS enzymes have been isolated and represent the products of three different genes. Two of the NOS enzymes, “neuronal NOS” (nNOS or NOS1) and “endothelial NOS” (eNOS or NOS3) were originally identified to be constitutively expressed and produce NO[•] at low amounts. Thus, the role of nNOS and eNOS was believed to be involved in the physiological function of NO[•] such as neurotransmission and vasodilation, respectively. The third NOS enzyme called the inducible or inflammatory NOS (iNOS or NOS2) was identified to produce NO[•] in response to a stimulus, mainly inflammatory stimuli, at high concentrations. Its role is believed to be involved in the pathophysiological functions of NO[•] such as antimicrobial effect, cellular injury and immunomodulation. However, recent studies have shown that NO[•] released from iNOS induction may also be

involved in physiological functions and that NO[•] released from nNOS or eNOS may be involved in pathological functions (15).

NO[•] is a radical, contains an unpaired electron and can form a covalent link with another molecule by sharing a pair of electrons. NO[•] can form nitroxyl anion (NO⁻) by accepting an electron or nitrosonium cation (NO⁺) by donating an electron. In living systems, NO⁻ can undergo spontaneous dimerization to form dinitrogen oxide (N₂O), or it can react with sulfhydryl groups (resulting in their oxidation) or with metals. NO⁺ is involved in nitrosation reactions with nucleophilic groups such as sulfhydryl groups, amides, carboxyls, hydroxyls, and aromatic rings (16).

Physiological effects of NO[•] and related species are, in most part, a result of the modification of the functions of many enzymes and other proteins. One of the first physiological role of NO[•] was identified as its ability to activate soluble guanylate cyclase *in vivo* (17). The neurotransmission and vasodilation actions of NO[•] are largely mediated by activation of guanylate cyclase after binding to its heme-group (18). Stimulation of guanylate cyclase leads to the synthesis of the biologically important second messenger, cyclic guanosine monophosphate (cGMP) and subsequent activation of cGMP-dependent kinases in responder cells. However, research over the last few years has identified physiological roles of NO[•] that are *independent* of cGMP (15, 19, 20). The majority of these involve the formation of S-nitrosothiols (RSNO) as a result of reaction between free sulfhydryl-containing molecules (RSH) and NO_x species. In proteins, reactive sulfhydryl groups are becoming regarded as a major intracellular target of NO_x. This post-translational modification has been shown to affect the activity of many important regulatory proteins, such as p21^{ras}, N-methyl-D-aspartate receptor, caspases, c-src and nuclear factor-κB (20, 21). This type of protein modification is increasingly becoming recognized as a ubiquitous regulatory reaction comparable to protein phosphorylation (20). In fact, RSNO may be responsible for some of the well-documented physiological processes that had been previously attributed to NO[•] itself, including activation of guanylate cyclase (19).

Pathological effects of NO[•] are mostly due to the deleterious NO_x species formed by the reaction of NO[•] with O₂, O₂^{•-} or other ROS (5, 11-13, 22). NO[•] can react with O₂ to form a powerful nitrosating agent nitrous anhydride (N₂O₃). This is thought to be a more reactive and damaging species than NO[•] itself. The chemistry of N₂O₃ has been extensively studied,

and its reaction with many compounds is known. It may form tertiary species, reacting with amines to form nitrosamines, or with sulfhydryl groups to form RSNO. N_2O_3 can also directly nitrosate the primary amines on DNA bases, ultimately leading to deamination. Any DNA base containing an exocyclic amino group can undergo deamination upon reaction with N_2O_3 . Therefore, adenine, cytosine, 5-methylcytosine, and guanine can all be deaminated to form hypoxanthine, uracil, thymine and xanthine, respectively (11, 22). As a consequence of these changes, point mutations may be induced in a cell by $NO^\bullet$ -related species. In addition, the ability of $NO^\bullet$ -derived species to induce large-scale mutations was also recently reported in mammalian cells (23).

Reaction of $NO^\bullet$ with $O_2^{\bullet-}$ rapidly produces peroxynitrite ($ONOO^-$), which is considered one of the most potent of the NO_x species involved in mediating cellular and tissue injury (24, 25). Formation of $ONOO^-$ is an extremely fast reaction due to the fact that both species are radicals. The reaction, depending upon the relative concentrations of $O_2^{\bullet-}$ and $NO^\bullet$, can be 3.5 times faster than the decomposition of $O_2^{\bullet-}$ by SOD (11). $ONOO^-$ is highly reactive at physiological pH, but when protonated it forms peroxynitrous acid that is a powerful oxidant with properties close to $OH^\bullet$. $ONOO^-$ has been shown to cause single strand breaks in DNA *in vitro* (11). In addition, $ONOO^-$ can directly damage DNA bases, but unlike N_2O_3 (which can react with adenine, guanine, and cytosine), $ONOO^-$ appears to significantly react only with guanine. Two main types of chemistries are attributed to $ONOO^-$, namely oxidations and nitrations. Thus, two main products have been identified from the reaction of $ONOO^-$ with deoxyguanosine (dG), namely 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) and 8-nitro-2'-deoxyguanosine (8-nitro-dG) (11). 8-oxo-dG has long been regarded as a biomarker for monitoring DNA damage in studies involving various ROS and $ONOO^-$, and its role in mutagenesis and carcinogenesis has been widely investigated. Several studies have shown a correlation between the formation of 8-oxo-dG and carcinogenesis. 8-nitro-dG was recently described and provides an attractive biomarker for detecting $ONOO^-$ -specific DNA damage, as nitration of DNA bases cannot be attributed to any other known types of damaging agents (26).

$ONOO^-$ is also involved in the oxidation and nitration of protein residues (such as, cysteine, tyrosine, methionine and other amino acids) (12). The main reaction of $ONOO^-$

with sulfhydryl groups of cysteine is an oxidation leading to disulfide formation, this being one of the mechanism of ONOO^- -mediated inactivation of various enzymes (including creatine kinase, α -1-antiproteinase, succinate dehydrogenase and fumarate reductase). The reaction between ONOO^- and cysteine groups, such as in protein or glutathione, can also lead to RSNO and RSNO_2 formation, especially in the presence of CO_2 . The most widely studied reaction of ONOO^- is with tyrosine residues in proteins leading to the formation of *nitrotyrosine* and di-tyrosine, as well as hydroxylated ring products (24, 25). The presence of nitrotyrosine-containing proteins in biological samples is extensively used as a molecular marker of reactive NO_x production in a tissue (27). Evidence for the role of ONOO^- *in vivo* is largely dependent upon the detection of nitrotyrosine in injured tissues. However, nitrotyrosine formation is not specific for ONOO^- . Nitrite (NO_2^-), a major end-product of $\text{NO}^\bullet$ metabolism, is converted by myeloperoxidase-catalyzed reactions into nitrogen dioxide ($\text{NO}_2^\bullet$) and nitryl chloride (NO_2Cl) that readily promote tyrosine nitration in a reaction independent of ONOO^- (28). Formation of nitrotyrosine residues in proteins can also alter function of some proteins (29-33), which may have both physiological and pathological significance.

Thus, the chemistry of $\text{NO}^\bullet$ is extremely complex due to the large number of chemical species formed and the numerous parallel reactions to consider. On one hand, $\text{NO}^\bullet$ -derived species can have physiological functions, such a vasodilation and neurotransmission. On the other hand, they can be pathological, as they are also capable of modifying/damaging DNA and proteins, as well as lipids. The ultimate effect of in $\text{NO}^\bullet$ a tissue depends upon the “nearby” reactive molecules.

v) *Inflammation and cancer: Inflammatory cells are a source of ROS/NO_x.*

A number of human cancers arise after a history of chronic inflammation. In fact, chronic inflammatory diseases may be a primary factor in the development of up to one-third of all human cancers (34). Diverse sources of inflammation may be effective including those caused by bacterial, viral and parasitic infections (Table I.3). Another major example of association of inflammation with cancer is in patients with inflammatory bowel disease, a series of chronic inflammatory disease of gastrointestinal tract (13). These include ulcerative colitis and Crohn’s disease. In the case of ulcerative colitis, 20-50% of patients with inflammation of 8 years or longer develop colonic cancer during their lifetime (4).

TABLE 1.3: Partial list of cancers that are believed to arise as a result of various infections.

	Infection	Cancer
Bacterial	<i>Helicobacter pylori</i>	Gastric (chronic gastritis/ulcer)
	<i>Salmonella typhi</i> , <i>S. paratyphi</i> A, B	Gallbladder cancer, pancreatic cancer
	Hepatitis virus Type B and type C	Hepatoma (chronic hepatitis/cirrhosis)
Viral	Herpes simplex virus type II	Cervical cancer (recurrent infections)
	Epstein-Barr virus	Gastric cancer, nasopharyngeal cancer (Burkitt lymphoma)
	Papilloma viruses	Cervical cancer
Parasitic	<i>Opisthorchis viverrini</i>	Cholangiocarcinoma (cholangiofibrosis)
	<i>Schistosoma</i> <i>haematobium</i>	Bladder cancer, hepatoma

From ref. (35).

The mechanism of inflammation-mediated cancer is believed to involve ROS and NO_x. Epidemiological studies have shown solid evidence of a link between formation of ROS/NO_x in tissue and carcinogenesis in the cases of ulcerative colitis, *H. pylori* and other infections (hepatitis B, HSV II, EBV, *O. viverrini*) (35). The presence of protein nitrotyrosine has been reported in inflammatory disorders such as *H. pylori* infection, Crohn's disease, ulcerative colitis, Wegener's granulomatosis, cystic fibrosis, asthma, obliterative bronchiolitis and rheumatoid arthritis (25, 29, 36-41). In addition, the level of protein nitrotyrosine has been found to correlate with the severity of inflammatory diseases (42-44).

Inflammatory cells such as neutrophils and macrophages can generate a large amount of ROS/NO_x, especially at the site of inflammation. Neutrophils are the first to arrive in large numbers at these sites, with other leukocytes being recruited later. Once neutrophils reach these sites, they are stimulated by cytokines to release (i) large amounts of ROS in a process termed "respiratory burst" and (ii) their granule contents (degranulation) (6). The respiratory burst first involves the assembly and activation of an enzymatic complex called NADH oxidase. This complex is able to generate O₂^{•-}, which can spontaneously or enzymatically (by SOD) be converted into H₂O₂. This can subsequently release the highly reactive OH[•]. However, the evidence for the formation of OH[•] *in vivo* is weak. The degranulation process involves the release of microbicidal molecules that are packed within the azurophilic granules of neutrophils. These include myeloperoxidase, serine proteases and antibiotic proteins (6). Myeloperoxidase is a neutrophil-specific enzyme and is also capable of generating ROS, as well as certain NO_x species. Myeloperoxidase-chloride system uses H₂O₂ to generate HOCl, which may further react with H₂O₂ to produce another mutagenic and cytotoxic ROS, singlet oxygen (¹O₂). Myeloperoxidase can also use nitrite (NO₂⁻), a major end-product of NO_x metabolism, to produce NO₂[•] and NO₂Cl, which readily promote nitrotyrosine formation (28). Macrophages are capable of producing large amounts of NO[•] at the sites of inflammation, as a result of the induction of iNOS. These amounts contribute to the microbicidal activity of macrophages (6). Although mouse neutrophils can also produce large amounts of NO[•] *via* iNOS, human neutrophils do not appear to contain iNOS (6). In addition, the microbicidal role of NO[•] in human neutrophils is controversial. These cells, however, do produce small amounts of NO[•], due to the presence of a "constitutive" NOS. In

addition, recent studies have shown that NO[•] released from constitutive NOS may also be involved in pathological functions (15). As discussed above, simultaneous release of ROS and NO[•] can lead to the formation of a significantly more damaging NO_x species, such as ONOO⁻. It has been reported that all 3 isoforms of NOS produce both NO[•] and O₂^{-•} simultaneously under certain conditions, especially when the substrate arginine is deficient (7-9). However, there is no evidence, so far, showing that ONOO⁻ is also formed *in vivo* by NOS. Thus, in mice, neutrophils-alone are capable of simultaneously releasing both ROS and NO[•] while, in humans, presence of both macrophages and neutrophils may be required.

Several groups have postulated that inflammation is the initiating factor in the development of many cancers (4, 13). It is believed that the potentially mutagenic ROS/NO_x released by inflammatory cells into the microenvironment of an inflamed tissue can lead to the induction of mutations. This is believed to contribute to the initiation of cancer. However, *the potentially mutagenic role of ROS/NO_x in the progression of cancer is not widely appreciated, even though the accumulation of mutations is believed to also play an important role in the later phases of cancer development.*

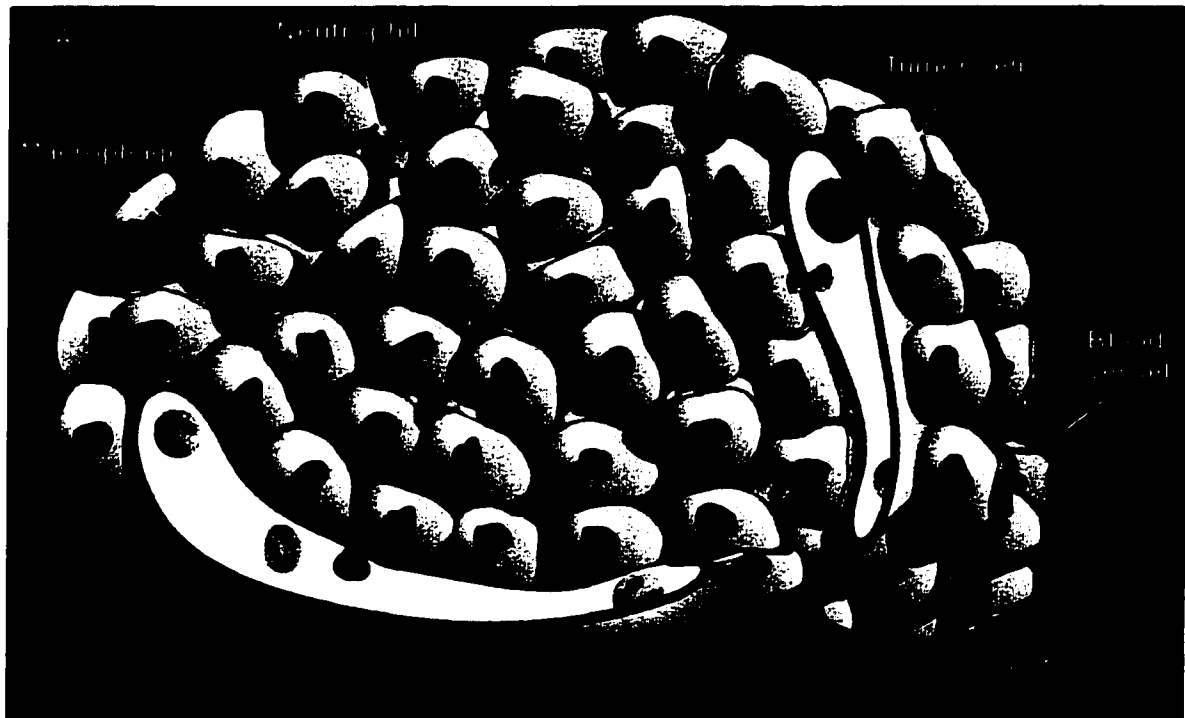
Many solid tumors in humans are infiltrated with inflammatory cells, such as neutrophils and macrophages (Fig. 1.2). Some of these include pulmonary adenocarcinoma, glioma, lymphoma, gastric carcinoma, melanoma, breast carcinoma and colorectal and prostate cancers (45-59). In many cases, the level of infiltration has been correlated with parameters associated with tumor progression. In addition, ROS/NO_x are known to be present in the tumor microenvironment. For example, protein nitrotyrosine has been detected in some tumors (60-63), and a high level of nitrotyrosine correlates with poor outcome in melanoma patients (64). *We and others have proposed that mutagenic ROS/NO_x generated by tumor-infiltrating inflammatory cells are in some measure responsible for contributing to the accumulation of mutations observed throughout tumor progression (65-68).*

vi) Enzymatic and non-enzymatic antioxidant systems

Antioxidants are an integral part of our defenses against reactive species. Biological antioxidants are scavengers of ROS/NO_x and include both enzymatic scavengers (e.g., SOD, catalase, glutathione peroxidase, glutathione reductase, formaldehyde dehydrogenase) and non-enzymatic scavengers (e.g., glutathione, vitamin E and C, uric acid). Most tissues can

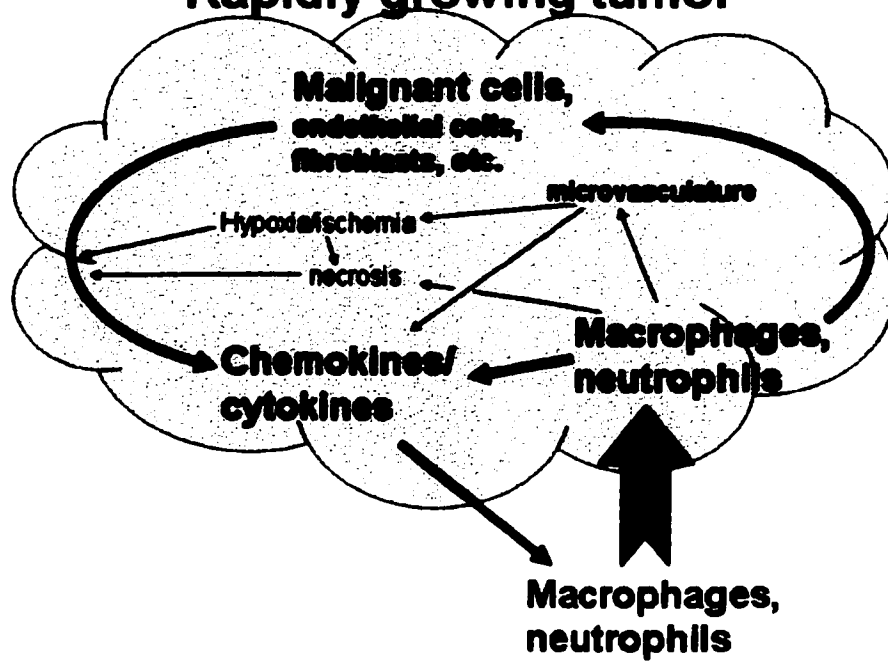
FIGURE 1.2. Inflammatory cell infiltration in solid tumors.

A) Schematic representation of a tumor infiltrated with inflammatory cells, mainly neutrophils and macrophages. **B)** A mechanism of inflammatory cell infiltration in a solid tumor. A rapidly growing tumor may consist of malignant cells as well as host cells (e.g., endothelial cells, fibroblasts). In some tumors these cells may directly release chemokines (e.g., interleukin-8) leading to recruitment of inflammatory cells. Most solid tumors have poorly developed microvasculature. It has been proposed that poor microvasculature of rapidly growing tumors fosters hypoxia, necrosis, and microthrombi (69, 70). These conditions can induce the nearby cells (e.g., tumor cells, endothelial cells) to generate chemotactic factors, promoting the infiltration of inflammatory cells. The recruited inflammatory cells may also release more chemotactic factors. In addition, products of inflammatory cells may cause damage to the tumor microvasculature and promote this cycle of events.



B

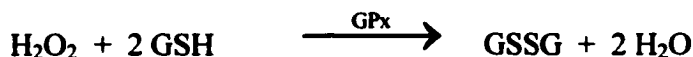
Rapidly growing tumor



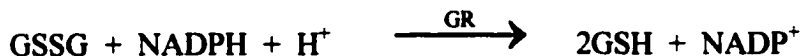
tolerate mild levels of ROS/NO_x, which often leads to the induction of defense systems. However, when generation of ROS/NO_x exceeds the capacity of antioxidants to scavenge them, tissue become vulnerable to damage leading to cell death and/or damage to DNA, lipids and proteins. There is abundant evidence both *in vitro* and *in vivo* that antioxidants protect against damage by ROS/NO_x and that depletion or low levels of antioxidants sensitizes towards the damage.

Glutathione (GSH) is an important component of cellular defenses against oxidants and xenobiotics (71). Intracellular GSH can be readily depleted using L-buthionine-(S,R)-sulfoximine, a drug that is a highly specific inhibitor of γ -glutamylcysteine synthetase. Depletion of cellular GSH has been shown to sensitize many cells to killing by various NO_x species (72-76). However, the mechanism of NO_x-mediated *mutagenicity* is not well understood. Our laboratory was the first to demonstrate the involvement of GSH in NO_x-mediated mutagenicity using the Mutatect cells *in vitro* (see below). Contrary to expectation, GSH was found to be pro-mutagenic, as it was actually associated with the induction of NO_x-mediated mutagenicity (77).

GSH is involved in both enzymatic and non-enzymatic ROS/NO_x-scavenging reactions. In enzymatic reactions, GSH usually acts as a cofactor for such enzymes as glutathione peroxidase (GPx, a selenium-containing enzyme), glutathione S-transferase or formaldehyde dehydrogenase (FDH). For example, GPx can detoxify H₂O₂ in cells by oxidizing GSH (78).

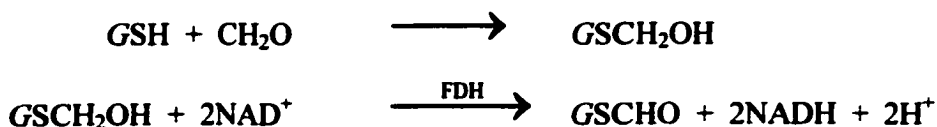


GSSG is then reduced to regenerate GSH by another important enzyme called glutathione reductase (GR) in the presence of NADPH (79).



These enzymes play an important role in scavenging ROS during oxidative damage. GSH also plays a key role in the FDH-catalyzed detoxification of formaldehyde (80-85), a cytotoxic and mutagenic species (86, 87). Humans are ubiquitously exposed to formaldehyde from exogenous sources, e.g., automotive emission, tobacco smoke, tissue fixatives, food and cosmetic preservatives and certain dental materials; in addition, formaldehyde is generated in cells by endogenous reactions e.g, demethylation reactions

involving serine and glycine metabolism (86). GSH readily reacts non-enzymatically with formaldehyde (CH₂O) to form *S*-hydroxymethylglutathione (GSCH₂OH), which is enzymatically oxidized into less toxic *S*-formylglutathione (GSCHO) by FDH.



The GSCHO is further detoxified by the enzyme *S*-formylglutathione hydrolase into carbon dioxide.

In non-enzymatic reactions, GSH directly reacts with various ROS or NO_x to produce less reactive species. For example, due to high selective reactivity of sulfhydryl groups towards NO_x (88), GSH readily reacts with many NO_x species to produce less reactive *S*-nitrosoglutathione (GSNO) (19, 89). GSNO was recently described to be rapidly detoxified in cells by FDH (90). The detoxification of GSNO by FDH is dependent upon the presence of NADH as well as GSH.



Thus, the presence of this GSNO-eliminating activity in FDH is believed to play an important role in detoxification of NO_x in cells (90).

Vitamin E is the most important non-enzymatic antioxidant in the prevention of cellular injury associated with ROS/NO_x (91, 92). Different forms of vitamin E are found in vegetable oils, nuts, wheat germ and grains of which D-α-tocopherol is considered to be the major biologically active form. Tocopherols are lipophilic molecules that have affinity for phospholipids in the plasma membranes, mitochondria and endoplasmic reticulum where they tend to concentrate and act as chain-breaking radical scavengers (93). The antioxidant activity of vitamin E has persuaded many groups to study its ability to prevent chronic diseases especially those believed to have oxidative stress (associated with ROS/NO_x) such as cardiovascular diseases, atherosclerosis, neurological disorders and cancer. Dietary supplements of vitamin E (in the form of D-α-tocopherol ester) have been shown to be significantly protective against cardiovascular diseases and atherosclerosis in humans and animals. In addition, overt vitamin E deficiency (occurring as a result of genetic

abnormalities in α -tocopherol transfer protein or as a result of various fat malabsorption syndromes) is associated with various neurological disorders and can be restored by vitamin E supplementation. Dietary supplements of vitamin E are widely self-administered in the belief that they can protect against cancer. However, clinical trials of vitamin E supplements or studies of disease risk associated with serum vitamin E levels have failed to show any consistently strong anticancer effect (94-104). An exception appears to be prostate cancer, where there is mounting evidence that vitamin E and other antioxidant micronutrients may be protective (100, 104-106).

b) Overall hypotheses

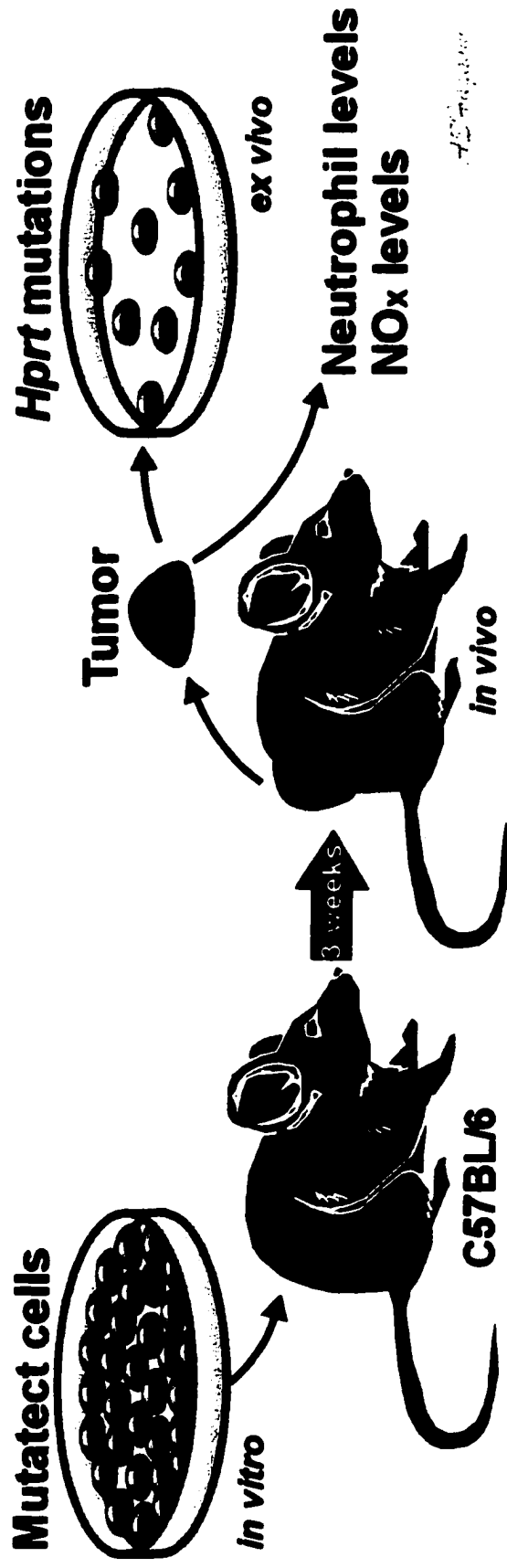
Overall hypothesis of our laboratory has been that inflammatory cells, which frequently infiltrate solid human tumors, are capable of generating ROS/NO_x that can induce mutations in tumor cells. We believe that this mutagenicity contributes to the accumulation of mutations observed throughout in tumor progression and can be reduced by various antioxidant systems.

c) The Mutatect system: an *in vitro*–*in vivo*–*ex vivo* mouse tumor model

The Mutatect¹ mouse model was developed in our laboratory to address the mutagenic role of factors present in the tumor microenvironment (66). The model permits detection of mutations arising in tumor cells *in vivo* from these factors at the hypoxanthine phosphoribosyltransferase (*hprt*) gene. Mutations at this locus are used as a non-selective surrogate marker of mutations occurring elsewhere in the genome. In Mutatect model, *in vitro*-growing Mutatect tumor cells are subcutaneously injected into syngeneic C57BL/6 mice, where they form a 1-cm tumor within 2-3 weeks (Fig. 1.3). The tumor is removed, lightly homogenized, and tumor cells are recovered and examined *ex vivo* for *hprt* mutations that arose *in vivo*. The tumor may also be used for studying the presence of inflammatory cell-infiltrates, generation of ROS/NO_x, and other parameters to address our hypothesis.

¹ Mutatect stands for “Mutation detect”

FIGURE 1.3. Mutatetect mouse tumor model



Mutatect cells were derived from a fibrosarcoma that was originally induced in a male C57BL mouse by a carcinogen methylcholanthrene (66). Cells were established in culture and genetically manipulated to develop a heterozygous *hprt* gene, suitable for sensitive detection of mutants. Mutations in this gene can be readily scored in a clonogenic assay because mutant cells are resistant to killing by cytotoxic drug, 6-thioguanine. The assay is capable of detecting as few as one *hprt* mutant cell in every 10^5 *hprt* wild-type cells. Mutatect cells have an aneuploid karyotype, typical of malignant cells. In particular, they have 3 copies of X chromosomes, even though the cells were derived from a male mouse. In the original Mutatect cell lines, all 3 X chromosomes appeared to be active, and it was therefore necessary to inactivate 2 copies of X-linked *hprt* locus. This created a heterozygous *hprt* gene ($-/-/+$) in a Mutatect cell line called MC17-51². Mutatect cells are sensitive to detection of *hprt* mutations because of the heterozygous state of the gene. Mutant cells in which large section of the genome is deleted, including a neighboring essential gene, will be viable because of the second or third copy of the essential gene on the remaining chromosome(s). Thus, the heterozygous gene permits detection of not only small, single-base mutations (or nucleotide sequence abnormalities) but also large, multi-locus deletion mutations (or chromosomal abnormalities). Both types of mutations are observed in human cancers (see *section i* above). The Mutatect MC17-51 cells were further manipulated into MN-11 cells by introducing a neomycin resistance gene (66). This allowed them to be distinguished from the host cells and more accurately quantify the levels of *hprt* mutation induced in Mutatect cells.

Several important findings have been observed by our laboratory using the Mutatect mouse model. The seminal observation was that tumor cells grown *in vivo* had a mean of 4-fold higher *hprt* mutation frequency than the same cells grown *in vitro* (66). This was the first direct quantitative evidence that the tumor microenvironment could indeed be mutagenic. This increase in mutation frequency has been a main focus in our laboratory, including work presented in this thesis. Although a mean of 4-fold higher mutation frequency was seen, a large variability among tumors was also seen; some showed no increase and some showed as high as 30-fold increase. When Mutatect tumors were examined histologically, frequent infiltration of inflammatory cells was observed (70). The

² Originally referred to as MC-TGS-17-51

most prominent of these cells was neutrophils, although presence of macrophages and lymphocytes was also sometimes observed. The extent of neutrophil infiltration was also observed to be variable among tumors. Microscopic counting of the neutrophils demonstrated a strong correlation between the number of neutrophils and *hprt* mutation frequency (70). Further examination showed that the neutrophils stained positively for iNOS (70), a source of NO[•] and related oxidants. In addition, there was a correlation between NOS activity and the *hprt* mutation frequency (70). Furthermore, the tumor cells stained positively for nitrotyrosine (70), a marker of NO_x. These results implicated a possible involvement of neutrophils and NO_x in the high *hprt* mutation frequency *in vivo*. To demonstrate that the neutrophils are in fact capable of inducing mutations, our laboratory attempted to increase the level of neutrophils in tumors. Interleukin-8 protein, a chemokine of neutrophils, was directly injected into established Mutatect tumors (70). This led to a significant increase in the neutrophil infiltration but only in 8 out of 36 (22%) of the interleukin-8-injected tumors (70). This was likely due to inconsistencies associated with interleukin-8 injections. Our laboratory has also shown that NO_x-donating drugs glyceryl trinitrate and molsidomine can induce a high *hprt* mutation frequency when administered to tumor-bearing animals (107). The effect of supplementing diets of tumor-bearing mice with vitamin E was also previously tested. A 25% reduction in the *hprt* mutation frequency *in vivo* was found in MN-11 tumors (107). This suggested a possible involvement of ROS/NO_x in the *in vivo*-arising mutagenicity. Thus, the Mutatect mouse model has been useful in implicating the role of tumor-infiltrating neutrophils and NO_x in the *in vivo*-arising mutagenicity.

The Mutatect system has also been useful in understanding the mechanism of NO_x-mediated mutagenicity (as well as cytotoxicity) in cultured cells. This is due to the high sensitivity of Mutatect cells to detection of *hprt* mutations. Mutagenicity and cytotoxicity of various ROS and NO_x species was previously described in Mutatect MN-11 cells *in vitro* (108). The NO_x donors glyceryl trinitrate and sodium nitroprusside were shown to induce high *hprt* mutation frequency in these cells. In addition, our laboratory was the first to demonstrate the involvement of GSH in NO_x-mediated mutagenicity using the Mutatect cells *in vitro* (77). Mutatect cells were first treated with L-buthionine-(S,R)-sulfoximine to deplete the intracellular GSH levels and then exposed to either sodium nitroprusside or glyceryl

trinitrate, to examine *hprt* mutation frequency. Although it was anticipated that the lack of GSH in cells would enhance the mutagenicity induced by the NO_x drugs, the opposite effect was observed. Depletion of GSH largely *prevented* the induction of *hprt* mutations by NO_x-donating drugs. A trivial explanation that the lack of GSH prevented bioactivation of these drugs was ruled out by showing that it had little effect on the accumulation of nitrite (NO₂⁻), a stable byproduct and one of a surrogate of NO_x production. Thus, this suggested that GSH is actually required for the induction of NO_x-mediated mutagenicity. To explain this, it was postulated that GSH reacts with NO_x to form GSNO that acts as a carrier of NO_x from cytoplasm to nucleus, liberating mutagenic species within the vicinity of DNA; when GSH levels are depleted, GSNO is not formed and mutations are not induced.

Many questions still needed to be answered to understand the roles and mechanisms of tumor-infiltrating neutrophils and reactive oxidants in relation to mutagenicity in Mutatect tumors. For example, could we consistently attract a high number of neutrophils into the tumors using interleukin-8? Are the tumor-infiltrating neutrophils, in fact, capable of inducing mutations in tumor cells? Are neutrophils inducing damage *via* NO_x in tumor cells? Is dietary vitamin E lowering the *in vivo*-arising *hprt* mutation frequency by scavenging NO_x species? Does GSH play a role as a transporter of NO_x species in the process of NO_x-mediated mutagenicity? These were some of the major questions that were addressed in this thesis. While attempting to answer them, several novel findings were also made.

d) Specific hypotheses

Based upon our overall hypothesis and the previous results obtained using the Mutatect system, the following hypotheses were proposed for the thesis:

- (1) A high number of neutrophils could be *consistently* attracted into Mutatect tumors if the tumors themselves are a source of interleukin-8, i.e., if the tumors are established by interleukin-8-producing cell lines.
- (2) Tumor infiltrating neutrophils can induce mutations in Mutatect tumors.
- (3) The level of protein nitrotyrosine would be higher in tumors with a high level of neutrophil infiltration, and the type of protein being nitrated might give us information into the mechanism of NO_x-mediated damage.

- (4) Dietary vitamin E would inhibit *hprt* mutation frequency and NO_x levels (estimated by nitrotyrosine) without affecting iNOS levels and neutrophil infiltration in Mutatect tumors.
- (5) The pro-mutagenic role of GSH involves the formation of intracellular GSNO, which acts as a carrier of NO_x in the Mutatect cells. The cells with high levels of GSH will have high levels of NO_x-mediated mutagenicity, while cells with low levels of GSH will have low levels of mutagenicity.

e) Specific objectives

To address the above hypotheses, our initial objectives were the followings:

- (1) To develop interleukin-8-producing Mutatect cell lines and demonstrate that they can still form tumors in C57BL/6 mice
- (2) To quantitatively measure neutrophil content in interleukin-8-producing Mutatect tumors
- (3) To measure *hprt* mutation frequency in interleukin-8-producing Mutatect tumors and demonstrate a correlation with neutrophil content
- (4) To compare the level of protein nitrotyrosine in interleukin-8-producing Mutatect tumors with non-producing tumors, and identify any prominent nitrated proteins by mass spectrometry
- (5) To examine the effects of vitamin E on *hprt* mutation frequency, neutrophil content, nitrotyrosine and iNOS levels in interleukin-8-producing Mutatect tumors
- (6) To genetically manipulate (down-regulate or up-regulate) GSH levels in Mutatect cells and examine the effect of NO_x-mediated mutagenicity (and cytotoxicity) in these cells
- (7) To measure intracellular formation of GSNO after exposure to NO_x-donors in Mutatect cells

f) Expected significance

Most of human tumors exhibit genetic instability, a feature characterized by accumulation of mutations. Over the last few decades, the sequence of events comprising cancer initiation and progression has been systematically unraveled, and it is now believed that multiple mutations are required to convert a normal cell into a malignant and a metastatic cell. Up to one-third of human cancers arise on a history of chronic inflammation,

and it is believed that inflammatory cell-derived mutagenic ROS/NO_x are involved in initial processes of cancer development. However, many types of solid tumors are also infiltrated with inflammatory cells, and the level of infiltration has been correlated with several parameters associated with tumor progression. *Mutagenicity induced in solid tumors by inflammatory cell-derived factors has not been previously reported.* Results presented in this thesis are expected to more directly support a role of tumor-infiltrating inflammatory cells and factors derived from these cells in genetic instability. The results are also expected to contribute towards understanding the mechanisms of NO_x-mediated genetic instability.

Interleukin-8-secreting Mutatect cell lines are expected to produce tumors with a consistently high level of neutrophils and consequently a high *hprt* mutation frequency. This is expected to directly demonstrate that neutrophils can induce genetic instability in tumors. It should also demonstrate associations among neutrophils, ROS/NO_x-damage (nitrotyrosine levels) and genetic instability. The long-term objective of these tumors is to study the effect of various dietary antioxidants (e.g., vitamin E) on genetic instability. Furthermore, examining the correlation between nitrotyrosine levels and mutation frequency in Mutatect model will help to establish its value as a surrogate marker for genetic damage in human tumor samples. Manipulation of GSH levels, and measurement of intracellular RSNO, should be useful in understanding the mechanisms of NO_x-mediated genetic instability.

Chapter 1. Construction of interleukin-8-secreting Mutatect cell lines and demonstration of their tumorigenicity

1.1. Chapter Summary

Mutatect cells are subcutaneously tumorigenic in syngeneic C57BL/6 mice since they grow into relatively large tumors within 3 weeks. To study the role of infiltrating neutrophils in tumors, our laboratory previously attempted to increase the levels of neutrophils by direct intratumoral injection of interleukin-8 protein, the most potent chemoattractant for neutrophils. However, these injections were not able to consistently chemoattract neutrophils. To provide a consistent source of interleukin-8 in tumors, interleukin-8-secreting Mutatect cell lines were developed. This chapter describes in detail the development of constitutive and tetracycline-regulatable interleukin-8-secreting cell lines and reports on their tumorigenicity. An interleukin-8 cDNA capable of expressing biologically active interleukin-8 protein was used as a starting point. One stable cell line, MIL-4, with high constitutive expression was isolated. MIL-4 cells produced very small, slow-growing subcutaneous tumors in C57BL/6 mice compared to non-interleukin-8-expressing controls. To address whether the lack of tumorigenicity was due to interleukin-8 expression, mixtures of MIL-4 and control cells were examined for tumorigenicity. Presence of MIL-4 cells in the mixture was found to be inhibitory to control cells in the tumors but not in culture. This suggested that factors, dependent upon the concentration of interleukin-8, in the tumor microenvironment are responsible for the lack of the tumorigenicity. Four stable Mutatect cell lines (TM-3, TM-7, TM-28, TM-34) with tetracycline-regulatable interleukin-8 expression were also developed. Level of interleukin-8 expression was high in TM-7 cells, low in TM-3 and TM-34 cells, and intermediate in TM-28 cells. Similar to MIL-4 cells, the TM-7 cells produced small, slow-growing tumors compared to lower expressors and non-expressors. Thus, tumorigenicity of interleukin-8-secreting Mutatect cells depends upon the level of interleukin-8 being secreted. Cell secreting very high levels produce very small tumors, whereas cells secreting lower levels produce large tumors. Level of neutrophil infiltration in these tumors is reported in *chapter 3*.

1.2. Introduction

Interleukin-8 (also known as neutrophil activating protein-1) is a pro-inflammatory chemokine (*chemotactic cytokine*) and one of the most potent chemo-attractant for neutrophils. It is expressed as a 99 amino acid protein that is secreted from cells after cleavage of a leader sequence of 27 residues. Interleukin-8 belongs to the CXC³ family of chemokines, which includes neutrophil activating protein-2, growth-related cytokines α , β and γ , and granulocyte chemotactic protein-2. These chemokines also act mainly upon neutrophils. A wide variety of cell types, including fibroblasts, endothelial cells, epithelial cells, monocytes, T lymphocytes and neutrophils can express interleukin-8. Expression of interleukin-8 usually occurs in response to injury of a tissue or infection by a pathogen and results in recruitment of neutrophils to that site. Specificity for neutrophils is a result of interleukin-8 binding to, and stimulation of, interleukin-8 receptors (CXCR-1 and -2), which are present predominantly on neutrophils. Neutrophils migrate along a concentration gradient of interleukin-8 and accumulate at the location of elevated concentration. Interleukin-8 is amongst the first of a set of cytokines that are produced in response to injury or infection. Thus, neutrophils are the first to arrive in large numbers at these sites, with other leukocytes being recruited later. Once neutrophils reach these sites, they are activated (by interleukin-8 or other cytokines) to produce a “respiratory burst” that generates cytotoxic and genotoxic species (e.g., $O_2^{\cdot-}$) and to release their stored lysosomal contents. This contributes to both host defense and tissue destruction and leads to pus formation seen in local sites of infection. Thus, interleukin-8 acts as one of the early mediators of inflammation by recruiting neutrophils to a site of tissue injury or infection by a pathogen.

Interleukin-8 also behaves as an angiogenic factor. It directly induces proliferation and chemotactic activities of endothelial cell *in vitro*, and angiogenesis *in vivo* in the absence of preceding inflammation (109). Angiogenic activity appears to be distinct from its ability to induce inflammation.

Persistent expression of interleukin-8 has been reported in many types of inflammatory disorders. These include *Helicobacter pylori* infection, Crohn’s disease, ulcerative colitis, rheumatoid arthritis and various types of lung-related inflammatory diseases. In addition, interleukin-8 is also present in many types of human tumors, including

³ CXC corresponds to first 2 cysteine residues separated by an amino acid.

melanoma (110, 111), non-small-cell lung tumors (112), bronchioloalveolar carcinoma (46), head and neck squamous carcinoma (113), gastric carcinomas (114), colon carcinoma (115), mesothelioma (116), and various glioblastoma (117, 118). In many of these diseases, interleukin-8 expression has been associated with a high level of neutrophil infiltration and increased vascularization, and in some cases with poor prognosis of the disease (46, 113, 114).

Interleukin-8 also has strong chemotactic activity for neutrophils from other species, including mouse, rat and monkey (119, 120). This characteristic has been utilized to explore the effect of interleukin-8 expression in several animal tumor models. However, paradoxical effects of interleukin-8 expression are seen. In some models, interleukin-8 secretion has been shown to be anti-tumorigenic. For example, interleukin-8 expression by Chinese hamster ovary cells in a nude mouse tumor model was shown to decrease subcutaneous tumorigenicity (121). Lack of tumorigenicity is usually associated with extensive infiltration of neutrophils (121, 122), which are known to generate cytotoxic factors (see *General Introduction*). However, in one case, an anti-tumorigenic effect independent of neutrophils was also observed to be associated with interleukin-8 expression (123). In other animal models, interleukin-8 expression was found to be pro-tumorigenic (124-126). For example, in human gastric carcinoma TMK-1 cells grown at an orthotopic site in nude mice, interleukin-8 expression from the cells produced rapidly growing and highly vascular tumors in the gastric wall (124). Thus, expression of interleukin-8 can either have anti- or pro-tumorigenic effects depending upon the animal model used or for other unidentified reasons.

Our laboratory has previously established the Mutatect tumor model as an experimental paradigm to study the effects of neutrophil-derived cytotoxic and mutagenic species on genetic instability in tumors (see *General Introduction*). The model consists of mouse fibrosarcoma cell lines that are subcutaneously tumorigenic in syngeneic C57BL/6 mice; they can grow into 1-cm tumors within 2-3 weeks after injection of 5×10^5 cells. The formation of relatively large tumors allows analysis of multiple parameters, including measurement of *hprt* mutation frequency and inflammatory cell content. Mutatect tumors are frequently infiltrated with host cells, most prominent of which are neutrophils (70). To study the effect of these tumor-infiltrating neutrophils on genetic instability, our laboratory initially attempted to increase the number of neutrophils in tumors. Interleukin-8 protein

was directly injected into established Mutatect tumors. This led to a significant increase in the neutrophil infiltration but only in 22% of the interleukin-8-injected tumors (i.e., 8 out of 36) (70). This was likely due to inconsistencies associated with interleukin-8 injections. In order to consistently attract a high number of neutrophils and provide a more biologically meaningful source of intratumoral interleukin-8, we set out to develop Mutatect cells that would be able to generate interleukin-8. Since it was believed that high level of interleukin-8 expression might lead to neutrophil-mediated anti-tumor effects, regulatable cell lines were also developed to reduce the high-level neutrophil recruitment. This chapter describes in detail the development of constitutive and tetracycline-regulatable interleukin-8-secreting cell lines and reports on their growth characteristics as subcutaneous tumors. The effect of interleukin-8 expression on neutrophil infiltration and genetic instability will be reported in *chapter 3*.

1.3. Hypotheses

The overall hypothesis was that neutrophils could be more consistently attracted into Mutatect tumors if the tumors themselves produce the chemokine interleukin-8, i.e., if the tumors are established by interleukin-8-producing Mutatect cell lines. However, the ability of these cell lines to form tumors (i.e., their tumorigenicity) would depend upon the concentration of interleukin-8 in the tumors. On one hand, very high levels of interleukin-8 may disrupt the formation of interleukin-8 concentration gradient, which is crucial in the directionality of neutrophil recruitment. On the other hand, high-level of uncontrollable production of interleukin-8 from Mutatect cells at the time of transplantation into mice may lead to extensive neutrophil-mediated cytotoxicity and inhibition of tumor establishment. However, if interleukin-8 production is very low, neutrophils may not be consistently chemoattracted.

1.4. Objectives and expected results

The overall objective was to develop interleukin-8-producing Mutatect cells and identify a cell line that is capable of *both* (i) forming large subcutaneous tumors (~1 cm diameter or > 50 mm³) in mice and (ii) consistently recruiting a high number of neutrophils

into tumors. The latter would be examined in *chapter 3*. The specific objectives addressed in this chapter are as follows:

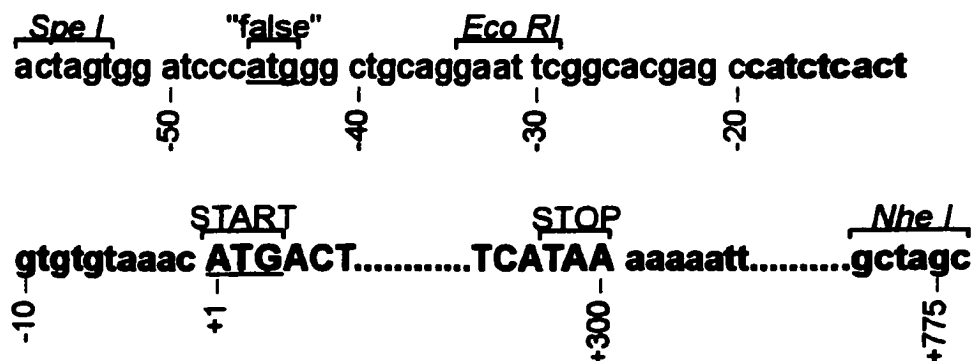
1. *To create a plasmid construct that expresses biologically active interleukin-8 protein.*
2. *To develop Mutatect cells that constitutively secrete interleukin-8 by stably transfecting the interleukin-8-expressing construct.* It was expected that clones secreting high, low and intermediate levels of interleukin-8 would be isolated.
3. *To demonstrate tumorigenicity of constitutive interleukin-8-secreting Mutatect cells in syngeneic C57BL/6 mice.* It was expected that constitutively excessive interleukin-8 expression at the time of tumor inoculation may result in early neutrophil-mediated cytotoxicity.
4. *To demonstrate tumorigenicity of mixtures of constitutive interleukin-8-secreting and non-secreting cells.* The mixture of cells was used in an attempt to lower the initial interleukin-8 concentration and was expected to enhance the tumorigenicity. .
5. *To develop Mutatect cells that secrete interleukin-8 in a regulatable fashion.* Since constitutive expression of interleukin-8 was expected to result in lack of tumor growth, a tetracycline-inducible expression system was also utilized. Several clones secreting high, low and intermediate levels of interleukin-8, in which the expression can be regulated at will, were expected to be isolated.
6. *To demonstrate tumorigenicity of regulatable interleukin-8-secreting Mutatect cells by inhibiting the interleukin-8 expression in the early stages of tumor growth.* It was expected that tumors formed in this manner would be larger than by tumors formed by the constitutive interleukin-8 expressing cells.

1.5. Materials and Methods

Reagents. All restriction endonucleases and DNA modifying enzymes used were from New England Biolabs Ltd. (Mississauga, Canada). Sulfosuccinimidyl biotin, *p*-nitrophenyl phosphate, fluorescamine, bovine serum albumin (BSA) and cetyltrimethylammonium bromide were from Sigma-Aldrich (Oakville, Canada). G-418 was from GIBCO-BRL (Burlington, Canada) and hygromycin was from Roche Diagnostics (Laval, Canada). Tetracycline hydrochloride administered to animals in the drinking water was diluted from a flavoured suspension for oral use (Novo-Tetra, Novopharm, Toronto,

Canada).

Construction of interleukin-8-expressing mammalian constructs. By searching in the Expressed Sequence Tag (EST) database (<http://www.ncbi.nlm.nih.gov/dbEST/index.html>), an I.M.A.G.E. Consortium LLNL cDNA (127) clone #328322 was identified to contain human interleukin-8 sequences. The clone was obtained from a commercial source (Research Genetics Inc., Huntsville, AL). To make a constitutive interleukin-8-expressing construct, a mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA) was used, which contains a cytomegalovirus promoter upstream of a multiple-cloning site. In the first attempt to make interleukin-8 expression constructs, clone #328322 was co-digested with Spe I and Nhe I to obtain an 833 bp fragment (see sequence below) containing the entire coding region of interleukin-8; the fragment was ligated to a compatible Xba I-digested pcDNA3, and a pcDNA3/IL8 clone L19-01 was identified to contain interleukin-8 under the cytomegalovirus promoter in the sense direction by restriction endonuclease-digestion analysis. However, this clone was unable to express interleukin-8 (see *Results* section) and, when sequenced, was found contain an additional “false” start codon that was 46 bp upstream of the authentic start codon of interleukin-8 coding region (see below).



The construct had to be re-created as follows. To avoid the sub-cloning of the “false” start codon, clone #328322 was first digested to completion with Nhe I, then partially digested with Eco RI, and finally blunt-ended with Mung bean nuclease to obtain an 810 bp fragment, containing the entire coding region of interleukin-8. The pcDNA3 vector was digested with Bam HI and blunt-ended with T4 DNA polymerase. The 833 bp fragment and the digested vector were ligated, and a pcDNA3/IL8 clone L25-13 was identified to contain the interleukin-8 coding region under the cytomegalovirus in a sense orientation by

restriction nuclease analysis (*Appendix A1*). The correct nucleotide sequence and existence of only one start codon was confirmed by sequencing from the T7 promoter of pcDNA3.

To make a regulatable interleukin-8-expressing construct, a mammalian expression vector pTRE (Clontech, Palo Alto, CA) was used since it contains a response element (TRE) for a tetracycline-responsive transcriptional activator (Clontech). To prepare a regulatable interleukin-8 construct, pcDNA3/IL8 clone L25-13 was digested with Bam HI and an 810 bp fragment, containing the entire coding region of interleukin-8, was isolated. The pTRE vector was also digested with Bam HI. The fragment and the vector were ligated and a pTRE-IL8 clone L1-2 was isolated (*Appendix A1*). It contained the interleukin-8 coding region in a sense orientation under TRE, as confirmed by restriction endonuclease analysis.

Cell culture conditions and interleukin-8 transfections. Unless otherwise stated, all cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10 % fetal calf serum (GIBCO- BRL, Burlington, Canada) in a humidified atmosphere of 5 % CO₂ at 37°C. The parental Mutatect cell line used for transfection experiments was MC-TGS17-51 (66) (referred to as MC17-51). All mammalian cell transfections in this chapter were carried out using DODAC:DOPE LuV liposomes according to manufacturer's protocol (INEX Pharmaceuticals Corp., Vancouver, Canada). For transient transfections, supercoiled plasmids were used, and culture media of transfected cells were analyzed after 48 h. For stable transfections, Sca I-linearized plasmids were used, and cells were transferred to either 0.5 mg/ml G-418 (a neomycin analog) or 0.3 mg/ml hygromycin after 48 h. Sca I cuts within the ampicillin gene of each plasmid. To produce constitutive interleukin-8-secreting stable cell-lines, MC17-51 cells were transfected with pcDNA3/IL8 clone L25-13, and G-418-resistant clones (named MIL clones) were selected. To produce regulatable interleukin-8-secreting stable cell-lines, the Tet-Off gene expression system (Clontech) was used. In the first step, linearized pTet-Off plasmid, containing genes for the tetracycline-responsive transcriptional activator (tTA) and neomycin resistance, was transfected into MC17-51 cells, and G-418-resistant clones (named MT clones) were selected. MT-6 clone, containing tetracycline-regulatable tTA activity, was identified as described in the *Results* section. In the second step, MT-6 cells were co-transfected with the linearized pTRE-IL8 clone L1-2 and a pTK-Hyg plasmid (Clontech), and hygromycin-resistant stable clones

(named TM clones) were selected. Cells were maintained in 10 µg/ml tetracycline-hydrochloride throughout the transfection. As described in *Results*, several interleukin-8 expressing TM clones were identified whose expression could be regulated by tetracycline.

Competitive ELISA for detection of interleukin-8 in culture medium. A competitive ELISA was developed to permit sensitive detection of interleukin-8 in the medium of Mutatect cells. Neutralite avidin (Molecular Probes, Leiden, Netherlands) was bound (50 µl per well of 20 µg/ml in PBS; room temperature for 18 h) to a 96-well polystyrene microtitre plate, then blocked with 1% BSA for 2 h. Recombinant human interleukin-8 (R & D Systems, Minneapolis, MN, supplied in solution as 300 µg/ml in 30 mg/ml BSA) was biotinylated as follows. A mixture was prepared containing 0.1 M sodium borate (pH 8.8), 50 µM sulfosuccinimidyl biotin, 0.6 µg recombinant interleukin-8 and 60 µg BSA in 200 µl and allowed to react overnight at room temperature. Unreacted functional groups of sulfosuccinimidyl biotin were blocked with 5 mM ammonium chloride at room temperature for 30 min. Free biotin was removed by extensive dialysis against PBS. As a control, biotinylated BSA was prepared similarly using a mixture lacking recombinant interleukin-8. Approximately 1 ng of dialyzed biotinylated-interleukin-8 in 100 µl of ELISA buffer (0.02 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05 % v/v Tween-20) was added per well of the avidin-coated, BSA-blocked 96-well plate and incubated at 4°C overnight. The wells were washed 3× with ELISA buffer and stored at 4°C for up to 1 month. Using anti-interleukin-8 antibody as described below, bound biotinylated-interleukin-8 could be detected while avidin-coated, BSA-blocked wells containing bound biotinylated-BSA, free recombinant interleukin-8 or no additive produced no signal.

Analysis of interleukin-8 in culture medium was carried out as follows. Cells were grown in DMEM plus 10 % fetal calf serum at a density of 5×10^4 cells per cm²; 18 h before analysis, the medium was removed and replaced with DMEM plus 0.1 % fetal calf serum. (Reducing the amount of serum was found to lower the background and increase the sensitivity of the ELISA; data not shown). The medium (0.5 ml) was mixed with rabbit anti-human interleukin-8 polyclonal antibody (0.5 µl of 1 mg/ml, Endogen, Woburn, MA) and incubated overnight at 4°C. Wells were washed before use with ELISA buffer, and 100 µl of culture medium preincubated with anti-interleukin-8 antibody was added and then incubated for 1 h at room temperature. Unbound antibody was removed by 3 washes with ELISA

buffer. Secondary antibody, 1:1000 diluted phosphatase-conjugated goat anti-rabbit immunoglobins (Kirkegaard & Perry, Gaithersburg, Maryland) (100 μ l per well) was added and incubated for 1 h at 37°C. Wells were washed 3 $\times$ with ELISA buffer, 100 μ l per well of substrate (3 mM *p*-nitrophenyl phosphate, 50 mM Na₂CO₃, 0.05 mM MgCl₂) was added and color was allowed to develop overnight at 4°C. Absorbance was measured at 405 nm in a Benchmark Microplate Reader (Bio-Rad, Mississauga, Canada). For correction of non-specific binding, the absorbance of wells without biotinylated-interleukin-8 was subtracted from the absorbance of corresponding biotinylated-interleukin-8-coated wells. A standard curve was constructed using 0 to 2 ng/ml of recombinant interleukin-8 added to DMEM plus 0.1 % fetal calf serum. Interleukin-8 produced by Mutatect cell-lines was quantified with reference to this standard curve. The assay was nearly linear in the range 0.02 to 1.50 ng of interleukin-8 per ml (data not shown). Each assay was performed in triplicate.

Biological activity of interleukin-8 produced by Mutatect cells. An *in vitro* chemotaxis assay for neutrophils was used to verify that human interleukin-8 produced by Mutatect cells had biological activity. Neutrophil-rich granulocytes were purified from the peripheral blood of normal healthy individuals using Ficoll-Hypaque (Pharmacia Biothec, Sweden) density gradient centrifugation followed by hypotonic lysis to remove erythrocytes (128). Culture medium (0.5 ml) from transiently transfected Mutatect cells was added to a 24-well plate. Nunc 10 mm Tissue Culture Inserts (Nalge Nunc International, Naperville, IL) with a 8 μ m polycarbonate membrane were placed into each well as the upper compartment of Boyden chamber (see Fig. 1.1B). A neutrophil-rich granulocyte suspension (0.4 ml containing 4×10^5 cells in PBS plus 0.9 mM CaCl₂, 0.5 mM MgCl₂, and 0.1% BSA) was added into each insert. The plate was incubated at 37°C for 60 min to permit chemotaxis of neutrophils through pores in the membrane. The membrane was removed, fixed in methanol, stained with hematoxylin and transferred to a glass cover slip. The membrane was air-dried and mounted on a glass slide with a drop of immersion oil such that the original lower surface of the membrane was uppermost. Neutrophils on this surface represent cells that have migrated through the pores of the membrane. The average number of neutrophils per $\times 400$ power field was calculated from a count of 8 fields. Experiments were carried out in duplicate.

Mutatect tumor formation and size measurements. Subcutaneous tumors were formed by injecting 5×10^5 cells in 0.1 ml of PBS into 8-10 week-old C57BL/6 female mice. Mixed tumors were formed by inoculating mixtures of MC17-51 and MIL-4 cells as indicated in the Table or Figure legends. For *in vitro* controls, the same cell mixtures were grown in culture for the entire period of *in vivo* tumor growth, with sub-culturing twice weekly. Where indicated, 0.4 mM tetracycline was added to the drinking water, which was protected from light and changed every 2-3 days. Once tumors reached a size where they could be measured with calipers (about one week after injection), three dimensions of each tumor were measured every 2 to 3 days. Tumor volumes were approximated by the following equation of a hemi-ellipsoid:

$$V = \frac{\pi \cdot l \cdot w \cdot h}{6}$$

where V is the volume of the tumor in mm^3 . Once the largest diameter of the control non-interleukin-8 producing tumors was ~1 cm (i.e., ~3 weeks), all animals were sacrificed. All animal experiments reported in this chapter were carried out at the Animal Care & Veterinary Service of the University of Ottawa in accordance with guidelines of the Canadian Council on Animal Care.

Statistical Analysis. For all statistical analyses, non-parametric tests were used. The Mann-Whitney U test was used to compare 2 unpaired groups. For 3 or more unpaired groups, a Kruskal-Wallis test was used. Spearman rank correlation was used to determine a correlation between 2 measured variables. Curve fitting and statistical tests were performed using GraphPad Prism version 3.02 (GraphPad Software, San Diego, CA). A value of $P < 0.05$ was considered to be statistically significant. In the figures, statistical significance is indicated as * (for $P < 0.05$), ** (for $P < 0.01$) or *** (for $P < 0.0001$).

1.6. Results

1.6.1. Development of a construct that expresses biologically active interleukin-8

A cDNA of human interleukin-8 was required as a starting point for making interleukin-8 expression constructs. By searching the EST database, a clone containing the

entire coding-region of human interleukin-8 cDNA was identified and obtained from a commercial source. To test its ability to express interleukin-8 protein, the coding-region was sub-cloned under a constitutively active cytomegalovirus promoter in a mammalian expression vector. This construct (clone L19-01) was then transiently transfected into Mutatect MC17-51 cells and the culture medium analyzed after 48 h for any secreted interleukin-8 using ELISA. No interleukin-8 was, however, detectable in the culture medium (Fig. 1.1A). When the interleukin-8 cDNA clone was sequenced, it was found to contain an additional start codon that was upstream of the authentic start of the interleukin-8 coding-region. Thus, the interleukin-8 construct had to be recreated to remove the additional “false” start codon (see *Materials and Methods* for details on reconstruction). The re-constructed plasmid (clone L25-13) was then transiently transfected into MC17-51 cells to test its ability to express interleukin-8 protein. Medium from the transfected cells was determined by ELISA to contain about 1.25 ng interleukin-8 per ml, whereas control medium (cell-free medium) or medium from vector-alone transfected cells contained < 0.1 ng/ml (Fig. 1.1A).

To test the biological activity of the expressed interleukin-8, an *in vitro* chemotaxis assay was carried out using a Boyden chamber (Fig. 1.1B). Culture medium from cells transfected with interleukin-8 construct was demonstrated to chemoattract a significantly higher number of human neutrophils than were the culture medium from vector-alone transfected cells ($P < 0.0001$) (Fig. 1.1C). These experiments demonstrate that the interleukin-8 construct (clone L25-13) is able to express biologically active interleukin-8 protein.

1.6.2. Construction of constitutive interleukin-8-secreting Mutatect cells (MIL-4)

Development of Mutatect cell lines that constitutively secrete interleukin-8 was accomplished by stable transfection of interleukin-8 construct (clone L25-13) into MC17-51 cells. Since this construct also contains a neomycin selectable marker gene, stable neomycin-resistant clones were isolated and screened for the secreted interleukin-8 levels in their cultured media by ELISA (Fig. 1.2). One clone, MIL-4 was found to consistently secrete 2.4 ng interleukin-8 / 10^6 cells / 24 h; others secreted (if any) below the detection limit < 0.05 ng / 10^6 cells / 24 h. The MIL-4 cells were used for further experiments as constitutive interleukin-8-secreting Mutatect cells, and the MIL-1 and the parental MC17-51

FIGURE 1.1. Mutatect MC17-51 cells, transiently transfected with a pcDNA3/IL8 construct (L25-13), express interleukin-8 that is biologically active.

Culture media from control (no cells), pcDNA3 (vector) or pcDNA-IL8 clone L25-13 or L19-01 transfected cells were examined. **A)** Interleukin-8 detected using a competitive ELISA. Only clone L25-13 produced interleukin-8 protein. **B)** Boyden chamber (an *in vitro* chemotaxis assay) used to measure biological activity of interleukin-8 for human neutrophils. **C)** Biological activity of interleukin-8 produced in the culture media measured using the Boyden chamber. Other details are in Materials and Methods. Each bar represents mean $\pm$ SEM of at least triplicate values. Differences between vector and L25-13 culture supernatants were highly statistically significant ($P < 0.0001$, indicated by ***). When normalized, 1 ng/ml interleukin-8 in the ELISA is equivalent to 7.5 ng per 10^6 cells per 24 h.

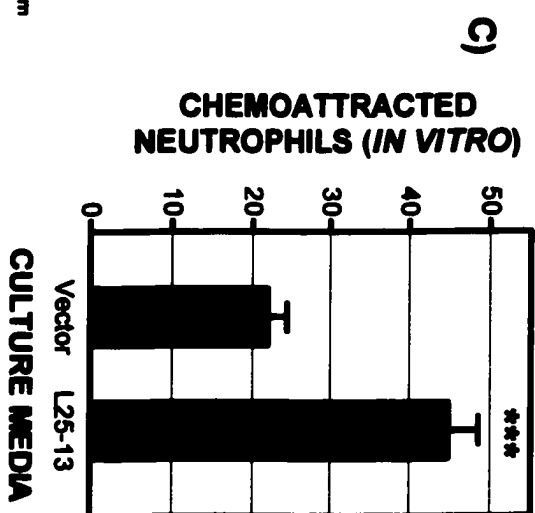
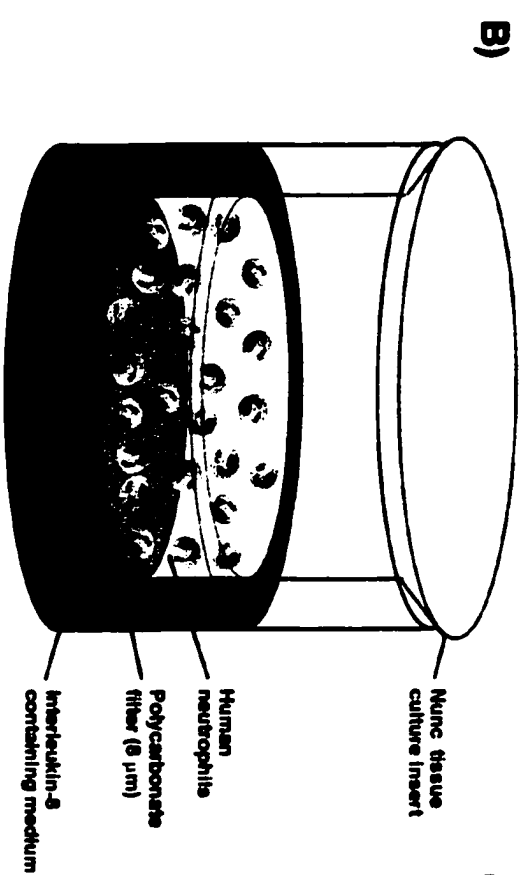
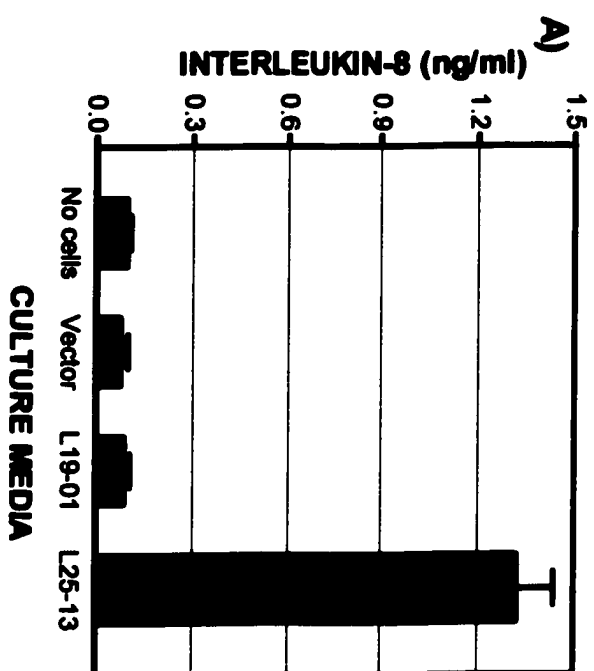
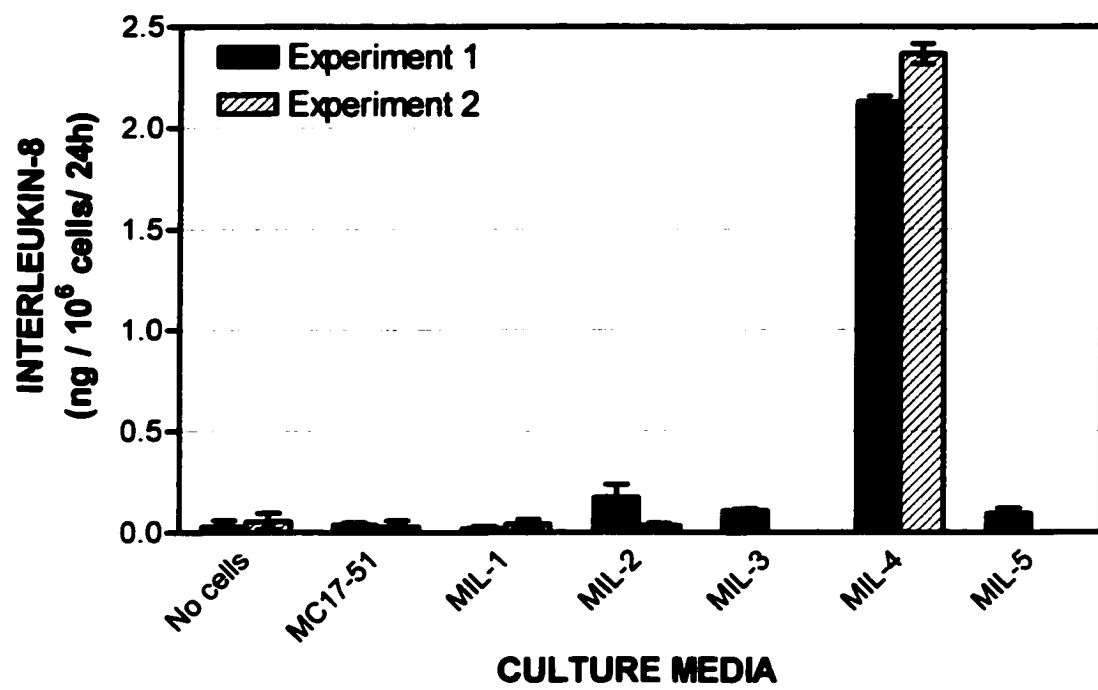


FIGURE 1.2. Interleukin-8 produced by neomycin-resistant MIL clones and controls. Mutatect MC17-51 cells were stably transfected with pcDNA3/IL8 clone L25-13 and neomycin-resistant MIL clones were isolated. Interleukin-8 in the culture medium (DMEM plus 0.1% fetal calf serum was substituted for complete medium for 24 h before analysis) was measured by ELISA. Other details are presented in Materials and Methods.



were used as control, non-interleukin-8-secreting cell lines.

1.6.3. Tumorigenicity of constitutive interleukin-8-secreting Mutatect MIL-4 cells

Mutatect cells are subcutaneously tumorigenic in syngeneic C57BL/6 mice since they can grow into 1-cm tumors within 2-3 weeks. To study the effect of constitutive interleukin-8 secretion on tumorigenicity of Mutatect cells, the growth of the interleukin-8-secreting MIL-4 cells was compared to the non-secreting MIL-1 and MC17-51 cells. Interleukin-8 secretion did not affect the *in vitro* growth rate of MIL-4 cells; all cell lines (MIL-4, MIL-1 or MC17-51) had doubling times of 14-16 h (Table 1.1). To determine their tumorigenicity and *in vivo* growth, 5×10^5 cells of each type were injected subcutaneously into groups of C57BL/6 mice and tumor size was measured at day 21. Mice injected with non-interleukin-8-secreting cells (MC17-51 or MIL-1) produced tumors that reached a size of about 1 cm in diameter at day 21 (Fig. 1.3A). By contrast, interleukin-8-secreting MIL-4 cells produced very small tumors, whose average size was < 0.3 cm diameter at day 21 ($P < 0.01$). In an independent experiment, growth rate of MIL-4 tumors was compared with that of MC17-51 tumors (Fig. 1.3B). Tumor volumes were monitored until excision at day 24. Doubling time of MIL-4 tumors was found to be 2.5 $\times$ higher than that of MC17-51 tumors (Table 1.1), in agreement with the above results (Fig. 1.3A). The growth kinetics of MIL-4 tumors indicated a lag phase that was at least 1 week longer than pure MC17-51 tumors. Thus, these results show that interleukin-8-secreting Mutatect cells are less tumorigenic since they produce small, slow-growing tumors compared to non-secreting cells.

1.6.4. Mixed tumors induced interleukin-8-secreting and non-secreting Mutatect cells

Slow growth of tumors initiated by interleukin-8-secreting MIL-4 cells may be due to the cytotoxicity by a high number of neutrophils expected to be attracted at the site of tumor injection. In an attempt to lower the local concentration of interleukin-8, we mixed interleukin-8-secreting MIL-4 cells with non-secreting MC17-51 cells prior to injection in the mice. In an initial experiment, the total number of cells injected was kept constant at 5×10^5 cells per injection, with varying ratios of the two cell types. As the number of MIL-4 cells in the tumors increased, the size of tumor became progressively smaller (Fig. 1.4A). This suggested that the low tumorigenicity of MIL-4 cells may be due to high interleukin-8

TABLE 1.1: Doubling times of constitutive and regulatable interleukin-8-secreting Mutatect cells growing in culture or as subcutaneous tumors in C57BL/6 mice

Cells	IL-8 ^a	Doubling time						
		Cells in culture (h) ^b			Tumor volume (d) ^c			
		Mean	± SD	n	Mean	± SD	n	
MC17-51	none	14.5	± 0.9	3	1.72	± 0.09	5	
MIL-1	none	15.3	± 2.1	3	ND			
Constitutive	MIL-4	high	14.3	± 1.6	3	4.27	± 0.78**	6
	MC17-51 : MIL-4 (0.5: 5)	low	ND			1.87	± 0.20	5
	MC17-51 : MIL-4 (1 : 5)	low	ND			1.82	± 0.22	5
	MC17-51 : MIL-4 (2 : 5)	low	ND			1.90	± 0.17	5
	MC17-51 : MIL-4 (4 : 5)	mod./low	ND			3.10	± 0.20*	5
Regulatable	MT-6	none	15.4	± 0.5	3	2.23	± 0.21	8
	TM-34	low	13.8	± 2.3	3	1.97	± 0.24	8
	TM-3	low	16.2	± 0.9	3	2.54	± 0.23	8
	TM-28	mod.	14.8	± 1.1	3	2.11	± 0.20	9
	TM-7	high	14.2	± 0.7	3	3.68	± 0.66*	8

^a Level of interleukin-8 secreted by cells. High >2, moderate 1–2, low 0.1–1 and none < 0.1 ng per 10⁶ cells per 24 h.

^b Cells growing on dishes were counted at time 0, 24, 48, 72 and 96 h using trypan blue-exclusion. The doubling time was calculated using the slope of the graph of Log (cell number) vs. time, using the equation doubling time = log 2 / slope.

^c Doubling time of tumors was measured as described in Fig. 1.3b.

ND - not determined

** p < 0.01 (Kruskal Wallis test)

* p < 0.05 (Kruskal Wallis test)

FIGURE 1.3. Comparison of tumorigenicity of Mutatect MIL-1, MIL-4 and MC17-51 cells in syngeneic C57BL/6 mice.

A) Tumor size (largest diameter) measured 21 days after subcutaneous injection of 5×10^5 MC17-51, MIL-1 or MIL-4 cells. **B)** Volume (V) of tumors at the indicated time following injection of MC17-51 or MIL-4 cells. The V was calculated as described in the Materials and Methods. **Inset)** A plot of $\text{Log } V$ against time to obtain the doubling time ($= \text{Log } 2 / \text{slope}$) of the tumors. Each point represents the mean volume of five tumors. Lines were curve fitted according to an exponential growth model using GraphPad Prism version 3.02 software.

levels, but it did not demonstrate the involvement of factors in the tumor microenvironment (likely neutrophils) in contributing to this low tumorigenicity.

To demonstrate whether the low tumorigenicity of MIL-4 cells was due to exogenous factors present in the tumor microenvironment, the ability of MIL-4 cells to act in *trans* to inhibit the tumorigenicity of MC17-51 cells was examined in a larger series involving 30 animals. An increasing number of MIL-4 cells (0, 0.5, 1, 2 or 4×10^5) was added to a constant number of MC17-51 cells (5×10^5), and each mixture was injected into 5 animals to allow subcutaneous tumor growth for 24 d. As an *in vitro* control, the mixtures were also grown in culture for an equivalent period. To study the effect of MIL-4 cell-addition *in vivo*, tumor sizes (volumes) were monitored throughout development. At day 24, the sizes of MIL-4/MC17-51 mixed tumors containing additional 1, 2 or 4×10^5 MIL-4 cells were significantly smaller than expected, whereas sizes of tumors containing additional 5×10^5 MC17-51 cells were similar to that expected (Fig 1.4B). In addition, tumors containing additional 4×10^5 MIL-4 cells had significantly lower growth rates than control tumors, as determined by an increase in the doubling time (Table 1.1). This suggested that addition of MIL-4 cells inhibited the growth of MC17-51 tumors.

To examine the effect of MIL-4 cell-addition on cell growth *in vitro*, the mixtures of MIL-4/MC17-51 cells growing in culture, which had not been passaged through the mice, were analyzed for neomycin resistance. This was done on the basis of the fact that MIL-4 cells are resistant to neomycin and MC17-51 cells are not (see section 1.5). As shown in Fig. 1.5A, the ratio of MC17-51 to MIL-4 cells in mixed cultures remained the same throughout the *in vitro* growth (> 3 weeks), suggesting that MIL-4 cells do not inhibit the growth of MC17-51 cells *in vitro*. Thus, the ability of interleukin-8-secreting MIL-4 cells to inhibit the growth of non-secreting MC17-51 cells *in vivo* but not *in vitro* suggests that factors dependent upon the concentration of interleukin-8 in the tumor microenvironment were likely responsible.

The tumors of MIL-4/MC17-51 mixture were also examined to determine whether either cell-type had a growth advantage *in vivo*. Tumors were analyzed for neomycin resistance to estimate the ratio of MIL-4:MC17-51. It was anticipated that the percentage of neomycin-resistant cells in the mixed tumors would decrease, since tumors initiated with 100% MIL-4 cells grew very slowly. Contrary to expectation, the number of neomycin-

FIGURE 1.4. Comparison of tumorigenicity of Mixtures of MIL-4 and MC17-51 cells in syngeneic C57BL/6 mice.

A) Tumor sizes of mixed tumors at day 20. The total number of cells injected were kept constant at 5×10^5 per injection, with varying ratios of the two cell types. **B)** Tumor volumes of mixed tumors at day 24. An increasing number of MIL-4 cells (0, 0.5, 1, 2 or 4) was added to a constant number of MC17-51 cells (5×10^5), and each mixture was injected into 5 animals. In 0:10 ratio, 10×10^5 of MC17-51 cells were injected. Each bar represents mean $\pm$ SEM of five tumors. The expected volumes were calculated based upon the volumes of pure MIL-4 and MC17-51 tumors and taking into account their ratios.

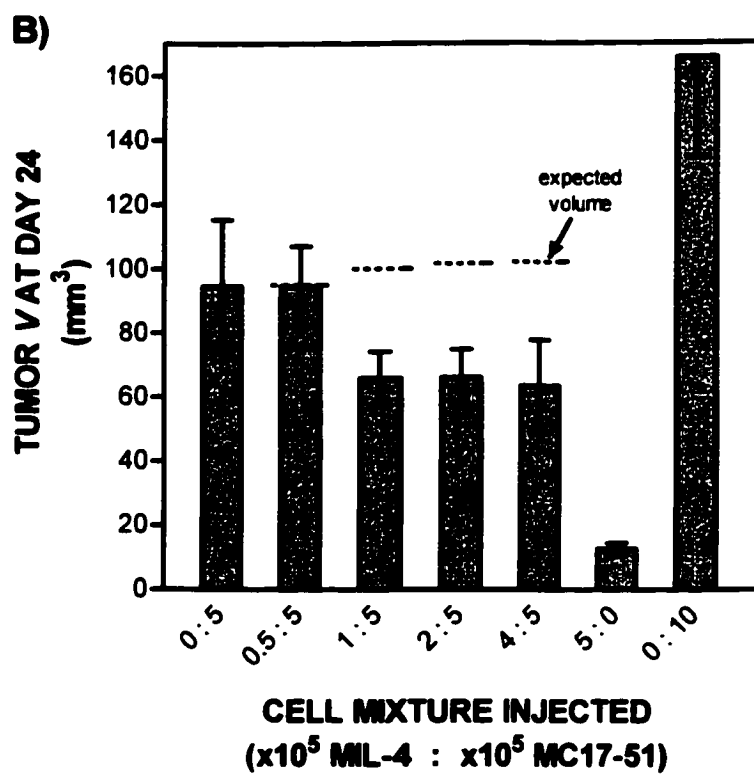
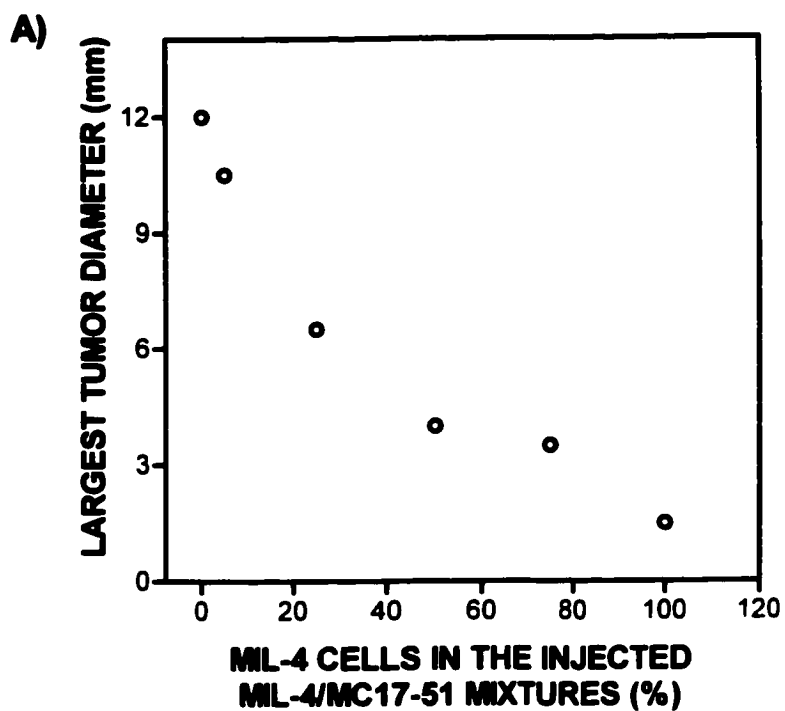
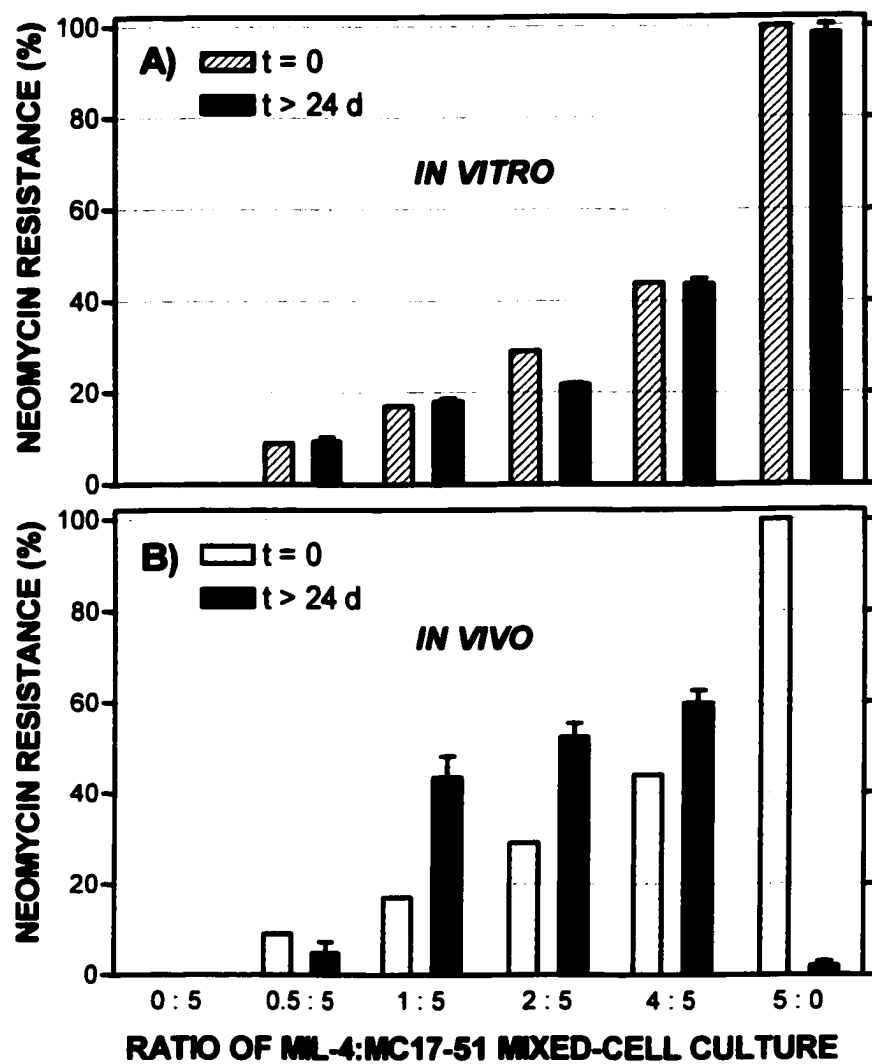


FIGURE 1.5. Expression of neomycin-resistant gene by Mixtures of MIL-4 and MC17-51 cells after 24 days compared to initial levels (t = 0).

A) Percentage of neomycin-resistant cells in the *in vitro*-growing mixtures. B) Percentage of neomycin-resistant cells in the cells recovered from mixed tumors. The values 0:5-to-5:0 refer to the ratio of MIL-4 and MC17-51 cells used for the mixtures as described in the legend of Fig. 1.4. Each bar represents mean $\pm$ SEM of 5 tumors or 3 cultures. Other details are in the Results section.



resistant cells recovered from mixed tumors was actually 20-100% higher than corresponding *in vitro* cultures in the case of 1:5, 2:5 and 4:5 ratios (Fig. 1.5B). This suggested a growth advantage to the interleukin-8-expressing MIL-4 cells in tumors under some concentrations of interleukin-8. Surprisingly, tumors initiated with 100% MIL-4 cells (the 5:0 ratio) were found to be >95% lower in neomycin resistance (Fig. 1.5B).

1.6.5. Construction of regulatable interleukin-8-secreting Mutatect cells (TM-28, TM-34, etc.)

Regulatable interleukin-8-secreting Mutatect cell lines were developed using a tetracycline-regulatable gene-expression system, Tet-Off™ from Clontech. The system consists of two plasmids (Fig. 1.6). One plasmid contains a response element (TRE) upstream the inserted gene of interest (e.g., interleukin-8). The other plasmid contains the gene for tetracycline-sensitive transcriptional activator (tTA) under a constitutively active cytomegalovirus promoter. The tTA binds to TRE and activates the downstream inserted genes in the absence but not in the presence of tetracycline (Fig. 1.6). The development of tetracycline-regulatable interleukin-8-secreting cell lines was accomplished in two steps. In the first step, Mutatect MC17-51 cells were transfected with the plasmid containing the tTA gene, and stable cell lines were isolated on the basis of a linked neomycin resistance gene. Fifteen stable neomycin-resistant clones, named MT clones, were selected. These clones were then screened for tTA activity as follows: each MT clone was transiently transfected with a TRE-regulated interleukin-8 construct and analyzed for secretion of interleukin-8 by ELISA (Fig 1.7A). Four MT clones were found to secrete detectable levels of interleukin-8 in the culture medium, indicating the presence of tTA activity. The tTA activity in these clones was then analyzed for sensitivity to tetracycline; each clone was transfected with TRE–interleukin-8 construct in the absence or presence of tetracycline and assayed for interleukin-8 in the culture media. Of all the clones tested, the MT-6 clone responded best to tetracycline (Fig. 1.7B). A dose-dependent response to tetracycline was also done on this clone. As low as 80 nM of tetracycline could completely inhibit interleukin-8 secretion in TRE–interleukin-8-transfected MT-6 cells (Fig. 1.7C). The MT-6 cells were chosen for the next step in creating regulatable interleukin-8-secreting Mutatect cell lines.

FIGURE 1.6. Tet-off gene expression system from Clontech.

In the absence of tetracycline, tTA is bound to TRE and activates transcription of downstream inserted genes (e.g., interleukin-8). In the presence of tetracycline, the tTA binds to tetracycline, changes conformation and is unable to bind to TRE. Hence, the downstream inserted gene is not transcribed.

Tet-Off gene expression system

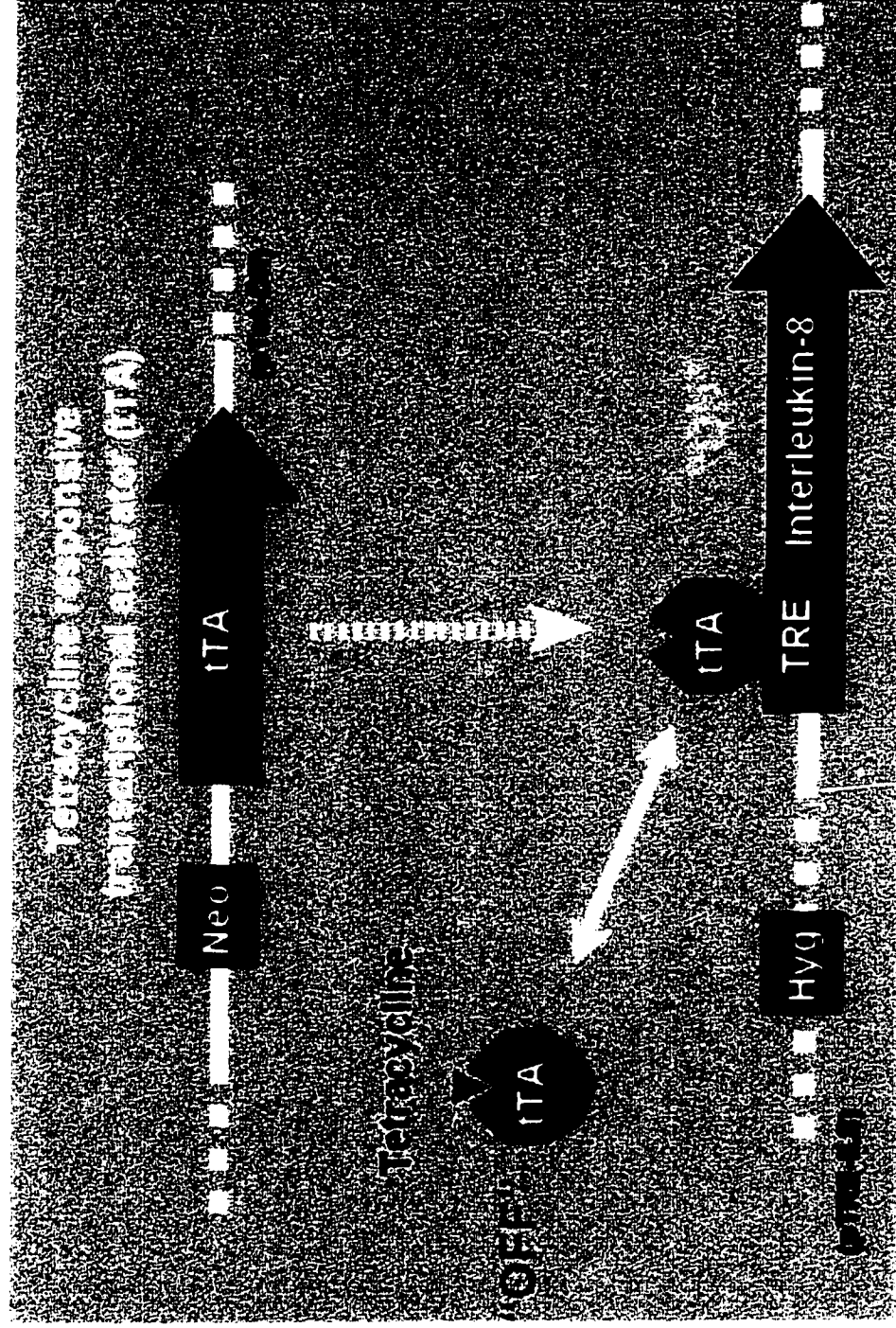
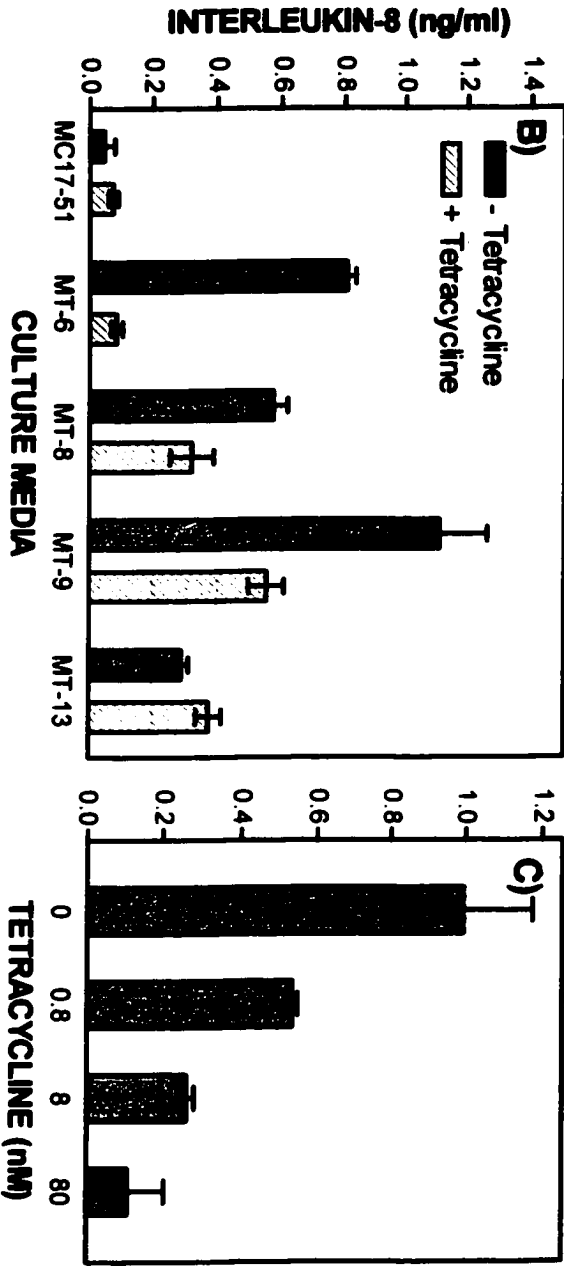
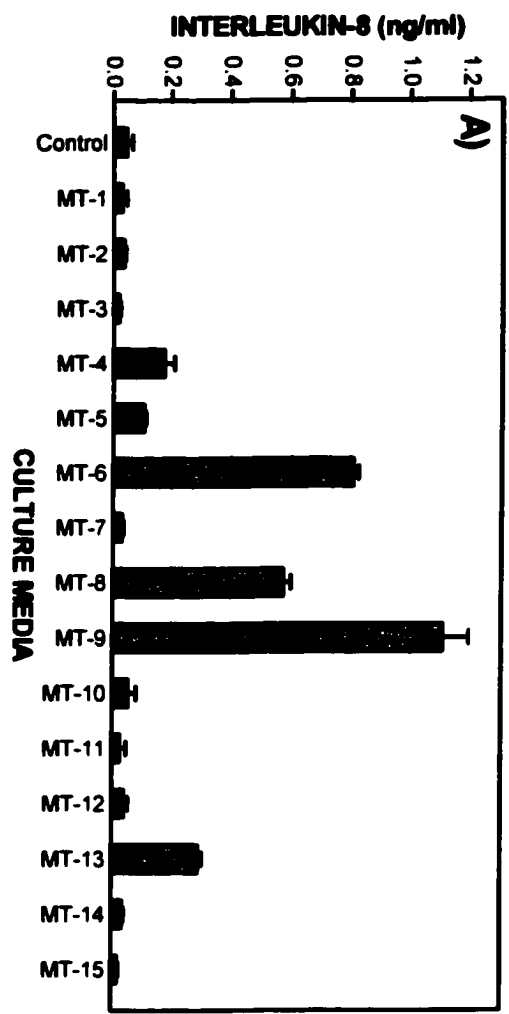


FIGURE 1.7. Mutatect MT clones.

Mutatect MC17-51 cells were stably transfected with pTET-Off plasmid and neomycin-resistant stable MT clones were isolated. **A)** Interleukin-8 produced by MT clones transiently transfected with TRE-interleukin-8 construct. **B)** Effect of tetracycline on interleukin-8 production by MT clones transiently transfected with TRE-interleukin-8 construct. **C)** Dose-dependent effect of tetracycline on interleukin-8 production by MT-6 clone transiently transfected with TRE-interleukin-8 construct. Interleukin-8 was measured by competitive ELISA. Each bar represents mean $\pm$ SD of triplicate values. Other details are in the Materials and Methods.



In the second step, MT-6 cells were co-transfected with the TRE–interleukin-8 construct and a hygromycin-resistance gene. Forty stable hygromycin-resistant clones, named TM clones, were isolated and their culture media screened for interleukin-8 using ELISA. Thirteen of the clones had detectable interleukin-8 levels (Fig. 1.8A). These were then analyzed for sensitivity to tetracycline; each clone was grown in the absence or presence of tetracycline and their culture media were assayed for interleukin-8. Only 4 clones exhibited stable interleukin-8 expression as well as effective tetracycline-dependent suppression of interleukin-8 (Fig. 1.8B). These 4 tetracycline-regulatable TM cell lines were chosen for further experiments, and the parental MT-6 cell line was used as a non-interleukin-8-expressing control cell line.

1.6.6. Tumorigenicity of regulatable interleukin-8-secreting Mutatect cells

The tumorigenicity of the regulatable interleukin-8-secreting Mutatect cells was studied by comparing the growth rates of the Mutatect TM clones 3, 7, 28 and 34 cells with that of the parental non-interleukin-8-secreting MT-6 cells. *In vitro*, all cell-lines had very similar growth rates in the absence (Table 1.1) or presence (data not shown) of tetracycline, with doubling times of 13-16 h. To measure growth rates *in vivo*, each cell line was transplanted into 8 mice and the tumor-volumes were monitored throughout development. Since results from *section 1.6.3* suggest that *constitutively* high interleukin-8-secreting cells produce tumors with a lag phase of one week longer than controls, all mice injected with TM cells were administered tetracycline in their drinking water for the first week in an attempt to suppress the initial interleukin-8 concentrations. After 1 week, half of the mice in each group were taken off the tetracycline, while the remainders were continued on the drug. No statistically significant difference in the tumor growth as a function of tetracycline for any clone was seen (data not shown). Tumors produced by interleukin-8-secreting TM 3, 28 and 34 cells did not differ significantly from control MT-6 tumors in their growth characteristics (Fig. 1.9A and Table 1.1). However, tumors produced by highest interleukin-8-secreting TM-7 cells were significantly smaller at day 19 than control MT-6 tumors ($P = 0.03$) (Fig 1.9A and Table 1.1). In addition, TM-7 tumors grew with similar growth kinetics (Fig 1.9B) as the tumors produced by high interleukin-8 secreting MIL-4 cells (Fig. 1.3B). Thus, cells expressing very high levels of interleukin-8 are appreciably less tumorigenic than cells expressing lower levels.

FIGURE 1.8. Interleukin-8 produced by stable Mutatect TM clones and control MT-6 clone.

A) Screen of interleukin-8 expression by TM clones. B) Tetracycline-responsive interleukin-8-secreting Mutatect TM clones. Cell were grown in the absence or presence of 1 μ M tetracycline and interleukin-8 was detected in the culture media by ELISA. MT-6 is a control non-interleukin-8 producing cell line. Error bars represent mean $\pm$ SEM of triplicate values.

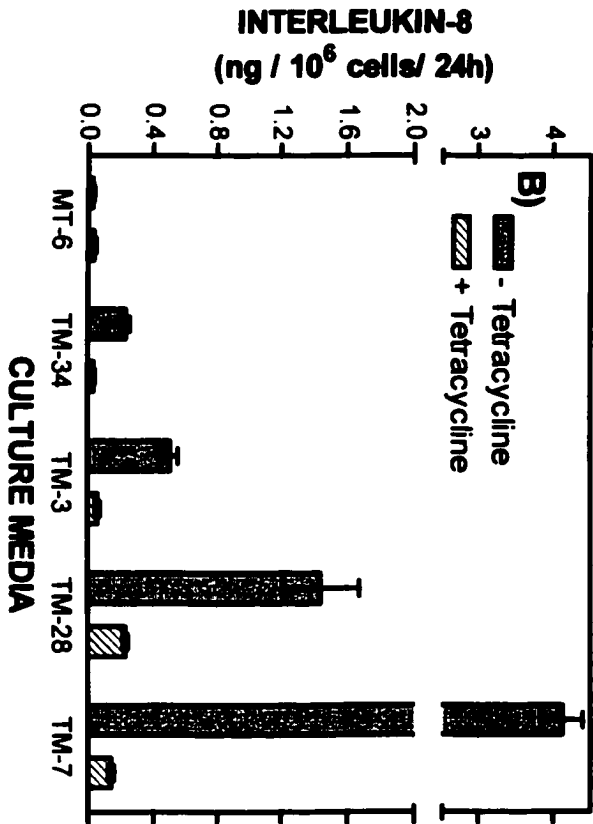
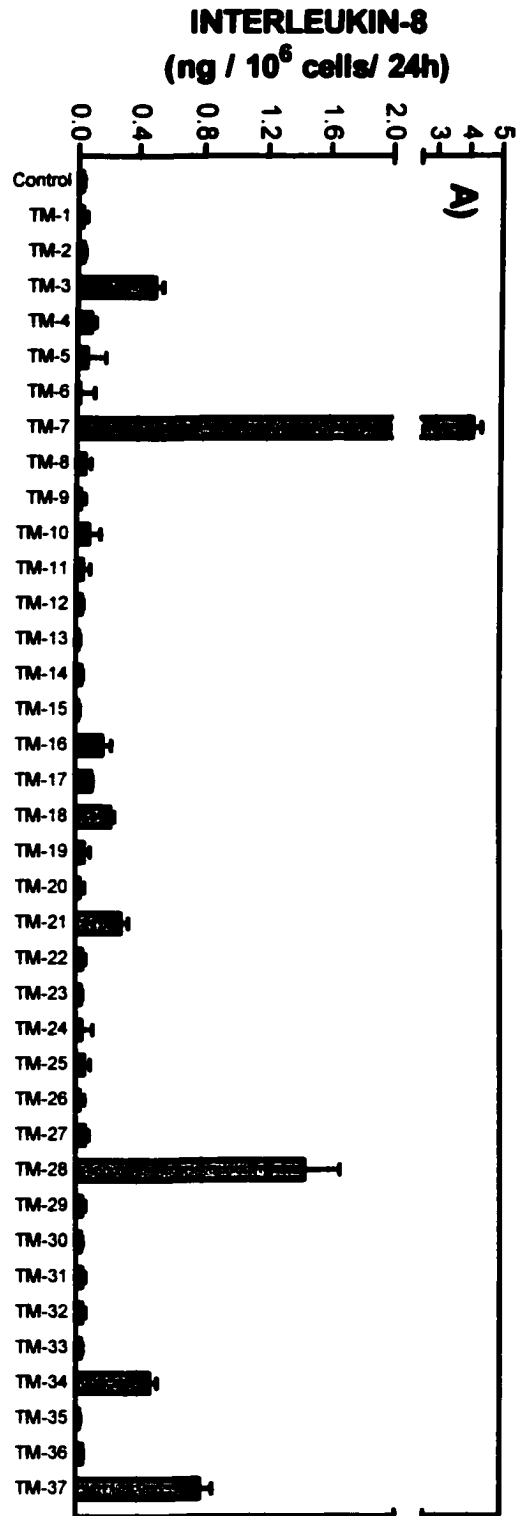
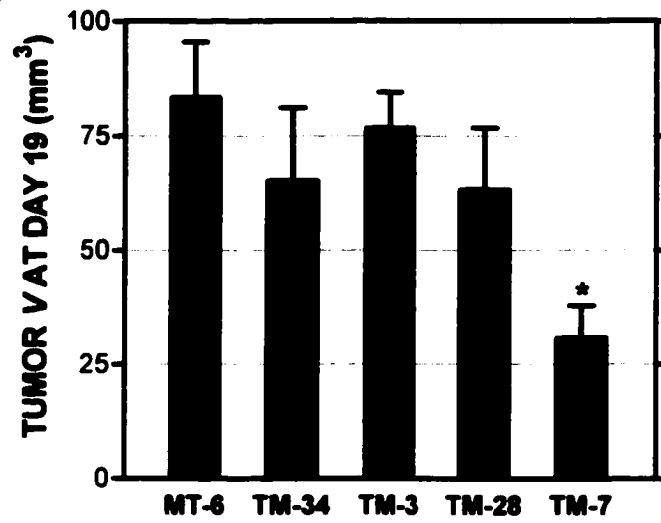


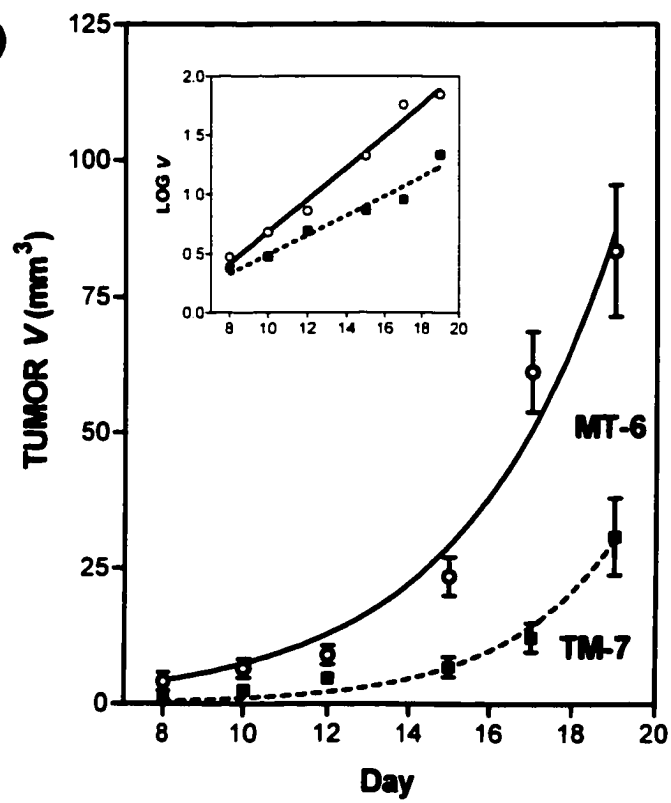
FIGURE 1.9. Comparison of tumorigenicity of Mutatect MT-6, TM-34, TM-3, TM-28 and TM-7 cells in syngeneic C57BL/6 mice.

A) Tumor volume measured 19 days after subcutaneous injection of 5×10^5 of the indicated cells. **B)** Volume (V) of tumors at the indicated time following injection of MT-6 control or TM-7, a high interleukin-8 expressor. The volume was calculated as described in the Materials and Methods. **Inset)** A plot of Log V against time to obtain the doubling time ($= \text{Log } 2 / \text{slope}$) of the tumors. Each point represents the mean $\pm$ SEM of 8 tumor volumes. Lines were curve-fitted according to an exponential growth model using GraphPad Prism version 3.02 software

A)



B)



1.7. Discussion

In several animal tumor models, interleukin-8 expression can result in extensive neutrophil-dependent or independent cytotoxicity, leading to very small-sized (if any) tumors (121, 122). Mutatect cells are subcutaneously tumorigenic in syngeneic C57BL/6 mice since they grow into 1-cm tumor diameter (or $\sim 90 \text{ mm}^3$ volume) within 3 weeks. These tumors can be analyzed for a number of parameters, including histological examination of sections, *hprt* mutation frequency, myeloperoxidase activity (chapter 2), interleukin-8 transgene instability (chapter 3), tocopherol levels (chapter 4), various immunohistochemical analyses (chapter 4) and/or protein nitrotyrosine (chapter 5). Multiple analyses are important to study associations amongst them in order to better understand the mechanism of genetic instability *in vivo*. Thus, it is desirable that the sizes of tumors be large enough ($> 50 \text{ mm}^3$) to complete most of these analyses. This chapter describes the development of interleukin-8-expressing Mutatect cell lines and identifies the ones that could produce large subcutaneous tumors.

As a starting point, an interleukin-8 cDNA was obtained. It was found to have an additional “false” start codon which needed to be removed to allow proper interleukin-8 expression. For it to be biologically active, the interleukin-8 protein needed to be properly processed before it is secreted from cells. Using ELISA and an *in vitro* chemotactic assay, we confirmed that the corrected interleukin-8 cDNA sequences were able to secrete biologically active interleukin-8 protein from Mutatect cells. This cDNA was used to make constitutive and regulatable interleukin-8-secreting cell lines.

The constitutive system was first developed and tested, since it was easier to construct and did not require many manipulations of Mutatect cells. Only one cell line, MIL-4, with constitutive interleukin-8 expression was isolated. These cells produced very small tumors ($< 20 \text{ mm}^3$) as they grew very slowly *in vivo* but not *in vitro*, suggesting that cytotoxic factors present in the tumor microenvironment may be responsible for the lack of tumorigenicity. In several other animal models, high levels of interleukin-8 expression can lead to neutrophil-dependent or, in one case, neutrophil-independent inhibition of tumorigenicity. Based upon this, we postulated that the lack of tumorigenicity of MIL-4 cells was due to high levels of interleukin-8 produced by the cells. We therefore attempted to lower the interleukin-8 levels by using mixed-cultures of interleukin-8-secreting MIL-4 and non-secreting MC17-51 cells to produce subcutaneous tumors. These tumors were

significantly larger ($>60 \text{ mm}^3$) than pure MIL-4 tumors, suggesting that the level of interleukin-8 was likely responsible for the lack of MIL-4 tumorigenicity. In most of the mixed tumors, MIL-4 cells were still a considerable part of the tumors as they accounted to 40-60% of the cells *in vivo*. These experiments also suggested that the lack of tumorigenicity is likely due to exogenous factors present in the tumor microenvironment, since the presence of MIL-4 cells in MIL-4/MC17-51 mixtures inhibited the growth of the mixtures *in vivo* (in the tumor microenvironment) but not *in vitro* (where cytotoxic factors are absent). Thus, from the constitutive system, we concluded that high interleukin-8-secreting Mutatect MIL-4 cells-alone produce small tumors, but when mixed with non-secreting MC17-51 cells to presumably reduce interleukin-8 levels, they produce larger tumors.

The fate of MIL-4 cells in MIL-4/MC17-51 mixed and pure MIL-4 tumors could be determined by the presence of neomycin resistance gene as a marker. In most ratios of mixed tumors, MIL-4 cells were enriched over MC17-51 cells. The enrichment of interleukin-8-secreting cells over non-secreting cells may be due to a growth advantage conferred by interleukin-8-mediated angiogenesis, although little angiogenic effect of interleukin-8 was seen. Surprisingly, in pure MIL-4 tumors, the neomycin resistance was lost, which was likely due to exogenous factors present in the tumor microenvironment (see *chapter 3*). Thus, the presence of interleukin-8-expressing cells in a tumor can have paradoxical effects. On one hand, it inhibits the growth of the tumor while, on the other hand, it gives a selective growth advantage to interleukin-8-secreting cells in the tumor. We believe that the ultimate effect of interleukin-8 on the biology of a tumor may depend upon its local *concentration*. At a very high concentration it may have anti-tumorigenic effects whereas at lower concentrations, the pro-tumorigenic effects would prevail.

In an attempt to completely suppress interleukin-8 expression from Mutatect cells, a more powerful genetic system was utilized. Interleukin-8 cDNA was placed under a tetracycline-regulatable promoter and used to construct Mutatect cell lines in which interleukin-8 expression could be down-regulated by tetracycline. This system required multiple steps and took longer to create than the constitutive system. At least 4 interleukin-8-secreting cell lines (TM-3, 7, 28, 34) were identified whose expression could be suppressed in cell culture by tetracycline. Clone TM-7 expressed the highest level of

interleukin-8, which was ~2-fold higher than MIL-4 cells. Clones TM-3 and TM-34 expressed low levels, while clone TM-28 expressed an intermediate level. These cells were transplanted into mice to examine their tumorigenicity. To avoid interleukin-8-dependent anti-tumorigenic effects, we attempted to suppress the initial expression of interleukin-8 by administering tetracycline to drinking water of mice for first 1 week. All the TM clones, with the exception of TM-7, produced large tumors ($>60 \text{ mm}^3$), which were comparable to the sizes of non-interleukin-8-secreting controls. In addition, TM-7 tumors appeared to be larger than MIL-4 tumors. However, continual administration of tetracycline throughout tumor development failed to affect tumorigenicity of any of the cell line. The effect of administering no tetracycline on tumorigenicity was not examined. Thus, we cannot conclude if tetracycline had any effects on tumorigenicity *in vivo*. However, what we can conclude is that cells containing very high levels of interleukin-8 (TM-7 and MIL-4) produce small tumors, while cells containing lower levels (TM-28, TM-3, TM-34 and mixture of MIL-4/MC17-51) produce large tumors.

1.8. Significance

Our laboratory has previously shown that interleukin-8 injected directly into Mutatect tumors was able to increase neutrophil infiltration. However, this was only seen in 22% of the injected tumors, which was likely due to inconsistencies with injections of interleukin-8. We have therefore developed interleukin-8-secreting Mutatect cell lines in an attempt to allow a consistent source of interleukin-8 in the tumors. The level of neutrophil infiltration in the tumors is reported in *chapter 3*. From the tumorigenicity studies, we have identified interleukin-8-secreting cell lines or mixture of cell lines that could produce large subcutaneous Mutatect tumors. These include TM-28, TM-3, TM-34 and mixture of MIL-4/MC17-51. It is desirable that the sizes of tumors be large enough in order to analyze these tumors for *multiple* analyses. These tumors were significantly useful in the latter chapters in studying the effects of neutrophils and neutrophil-derived reactive nitrogen oxide species on genetic instability *in vivo* (*chapters 3, 4 and 5*). They were analyzed for a number of parameters, including histological examination of sections (*chapter 3*), *hprt* mutation frequency (*chapter 3*), myeloperoxidase activity (*chapter 2*), interleukin-8 transgene instability (*chapter 3*), tocopherol levels (*chapter 4*), various immunohistochemical analyses

(chapter 4) and/or protein nitrotyrosine by Western blotting and mass spectrometry (chapter 5).

Finally, our results may explain the paradoxical effects of interleukin-8 in other animal tumor models. In some models it can have anti-tumorigenic effects while in others it has pro-tumorigenic effects. Although an obvious explanation for this difference is the different genetic makeup of the cell lines used in the different models, our results implicate that the ultimate effect of interleukin-8 on the biology of a tumor may also depend upon its local concentration. At a very high concentration, it has anti-tumorigenic effects whereas at lower concentrations, the pro-tumorigenic effects appear to prevail.

1.9. Conclusions

Tumorigenicity of interleukin-8-secreting Mutatect cells depends upon the level of interleukin-8 being secreted. Cells secreting very high levels produce very small tumors ($<50 \text{ mm}^3$), whereas the cells secreting lower levels produce large tumors ($>60 \text{ mm}^3$). TM-28, TM-3, TM-34 and mixture of MIL-4/MC17-51 were identified as the ones capable of forming large subcutaneous tumors.

Chapter 2. Development of a biochemical assay to assess neutrophil content in Mutatect tumors

2.1. Chapter Summary

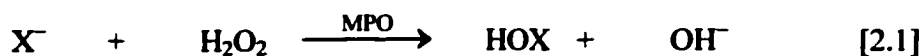
A quantitative method was necessary to reliably measure neutrophil infiltration in Mutatect tumors. Myeloperoxidase is a neutrophil-specific protein. Its enzymatic activity is often used as a marker of neutrophil infiltration into tissues. However, we and others have found that most enzymatic assays for myeloperoxidase are susceptible to interference from non-specific peroxidases as well as heme-containing proteins present in tissues. This chapter describes the development of a sensitive myeloperoxidase-specific assay, based upon bromide-dependent chemiluminescence of luminol, which can be used to reliably quantify neutrophil content in Mutatect tumors. The assay can distinguish between myeloperoxidase-specific and non-specific reactions. The myeloperoxidase-specific reaction is believed to proceed in 2 steps: (i) the enzymatic generation of hypobromous acid from bromide and H_2O_2 at pH 5, and (ii) the spontaneous reaction of hypobromous acid and H_2O_2 with luminol to give a chemiluminescence signal. The assay is sufficiently sensitive to allow detection of myeloperoxidase in <100 human neutrophils. Other peroxidases and hemoproteins do not interfere with the bromide-dependent signal. Although eosinophil peroxidase (a form of myeloperoxidase present in eosinophils) can also oxidize bromide to generate hypobromous acid, activities of neutrophil myeloperoxidase and eosinophil peroxidase can be distinguished at different pHs. As a demonstration of the utility of our bromide-dependent assay, myeloperoxidase activity was measured in Mutatect tumors. A statistically significant correlation was seen between myeloperoxidase activity and histological neutrophil counts in the tumors ($r = 0.69$, $p < 0.01$, $n = 14$). In addition, the assay was shown to be more reliable than the neutrophil counts in predicting *hprt* mutation frequency in tumors. The assay also has wider application for measuring the neutrophil content of other tissues.

2.2. Introduction

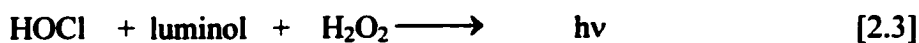
Neutrophil infiltration is frequently observed in Mutatect tumors (70). Interleukin-8-secreting Mutatect tumors were described in the previous chapter; the neutrophil infiltration in these tumors is expected to be higher. Quantification of neutrophils is important in order to compare with other quantified parameters, such as the number of mutations occurring at a marker *hprt* locus. The presence of neutrophils can be detected by histological examination of tumor sections, and the number of neutrophils in each section can be quantified by counting microscopically (70). However, this is a very tedious method that is semi-quantitative at best. Furthermore, neutrophils may be distributed very heterogeneously in tumors, making counts less reliable. Thus, to reliably estimate neutrophil content in a whole tumor, we utilized a biochemical assay to measure enzymatic activity of myeloperoxidase, a neutrophil-specific protein.

Myeloperoxidase (EC 1.11.1.7) is abundantly present in storage granules of neutrophils. It is widely considered a neutrophil-specific marker (129-133), although very low levels of myeloperoxidase are also found in monocytes (<5% the amount found in neutrophils, on a per cell basis) (134-136). Biochemical assays that measure myeloperoxidase activity are used to estimate neutrophil infiltration. There are 2 types of assays that are most commonly used: spectrophotometric and chemiluminescence assays. Both types of assays involve myeloperoxidase-catalyzed oxidation of the substrates in the presence of H₂O₂. Spectrophotometric assays commonly use substrates such as tetramethylbenzidine, guaiacol or *o*-dianisidine (137-139). Chemiluminescence assays use luminol as substrate at pH 7 (140-142) and are appreciably more sensitive than spectrophotometric assays. However, none of the assays is very specific for neutrophil myeloperoxidase. Heme-containing proteins (such as hemoglobin and myoglobin), and other tissue peroxidases can also catalyze oxidation of the substrates in the presence of H₂O₂ (143). In addition, eosinophil peroxidase, a myeloperoxidase-like enzyme present in eosinophils, may also contribute to the background signal (143). Thus, the widely used biochemical assays are unable to distinguish between the myeloperoxidase-specific and non-specific signals. Because of a requirement for a myeloperoxidase-specific assay to study neutrophil content in Mutatect tumors, the method described in the present chapter was developed.

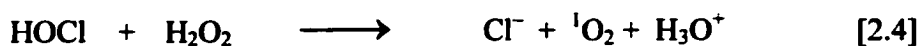
A unique property of myeloperoxidases is their ability to catalyze H₂O₂-mediated oxidation of halides (X⁻ = Cl⁻, Br⁻) to form hypohalous acids (HOX) (144, 145).



Most luminol-based chemiluminescence assays for myeloperoxidase are based upon the myeloperoxidase-H₂O₂-chloride system to form HOCl at neutral pH (equation 2.2). The HOCl can in turn react with luminol and H₂O₂ to give chemiluminescence (144, 145)



The HOCl (and other HOX) may also react with H₂O₂-alone to generate singlet oxygen (¹O₂).



In fact, ¹O₂ levels produced by myeloperoxidases are useful in evaluating the various myeloperoxidase-H₂O₂-halide systems. Kanofsky *et al* have shown that the myeloperoxidase-H₂O₂-*bromide* system is able to produce ¹O₂ at all pH tested (pH 3-8) (10, 146). However, under acidic conditions (pH 3-6) the myeloperoxidase-H₂O₂-*chloride* system is unable to produce any ¹O₂ (10, 146). In addition, under acidic conditions, neutrophil myeloperoxidase are much more active than eosinophil peroxidase (146). Based upon these differences, we describe a bromide-dependent chemiluminescence assay and demonstrate its specificity for neutrophil myeloperoxidase at pH 5. To demonstrate the utility of the assay, we used it to measure neutrophil myeloperoxidase in Mutatect tumors and correlated this measurement with the number of infiltrating neutrophil as assessed microscopically.

2.3. Hypotheses

The myeloperoxidase-specific signals can be distinguished from non-specific signals in a luminol-dependent chemiluminescence assay based upon the unique property of the neutrophil myeloperoxidase-H₂O₂-bromide system at acidic pH 5.

2.4. Objectives and expected results

The following were the specific objectives examined in this chapter:

1. *To design a two-step chemiluminescence assay that is able to distinguish non-specific signal (step 1) from bromide-dependent myeloperoxidase-specific signal (step 2).*
Human neutrophil and HL-60 cell extracts were used as positive controls for myeloperoxidase, while Mutatect and 293T cells were used as negative controls.
2. *To determine the linearity and detection limit of the assay using human neutrophil extracts.*
3. *To demonstrate the specificity of the assay by demonstrating any interference by heme-containing proteins or non-specific peroxidases.* It was expected that heme-containing proteins or non-specific peroxidases would contribute to the non-specific signal but not to the bromide-dependent signal.
4. *To distinguish between eosinophil peroxidase and myeloperoxidase signals using the bromide-dependent assay.* We expected to distinguish between the two peroxidases by carrying out the assay at two pH 5 and 7.5, since the myeloperoxidase-H₂O₂-bromide system is more active than the eosinophil peroxidase-H₂O₂-bromide system in acidic conditions (146).
5. *To demonstrate the ability of the bromide-dependent assay to quantitatively measure neutrophil content in Mutatect tumors and compare with histologically counted neutrophil content.*

2.5. Materials and Methods

Reagents. Hypochlorous acid (HOCl) was prepared from a 5.25 % commercial bleach solution (Javex) that was acidified to pH 8. Hypobromous acid (HOBr) solution was prepared by the addition of HOCl to buffer containing excess KBr; this solution is an

equilibrium of HOBr, bromine, and tribromide anion (10). These solutions were prepared immediately before use. Hexadecyltrimethylammonium bromide (CETAB), diethylenetriaminepentaacetic acid (DTPA), 3-amino-1,2,4-triazole (ATA), luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and MOPS buffer were from Sigma-Aldrich (Oakville, ON, Canada).

Isolation of peripheral blood granulocytes from normal human donors. Neutrophil-rich granulocytes were purified from the peripheral blood of normal healthy individuals using Ficoll-Hypaque (Pharmacia Biothech, Sweden) density gradient centrifugation followed by hypotonic lysis to remove erythrocytes (128). Purified granulocytes were cytocentrifuged (5 min at 500 rpm) onto glass slides and stained with Giemsa. Typical preparations contained > 98% granulocytes (about 96 % of which are neutrophils and about 4 % were eosinophils).

Isolation of human eosinophil-rich granulocyte preparations. Peripheral blood granulocytes were isolated from human patients with unspecified allergic conditions characterized by high eosinophil counts at Ottawa hospital, General campus; Ficoll-Hypaque density gradient centrifugation was used as described above. The mononuclear cell layer was discarded and the upper third of the erythrocyte/granulocyte layer (richest in eosinophils) was isolated. In 3 patients, these granulocyte preparations consisted of 43 %, 35 %, and 23 % eosinophils and 57 %, 65 %, and 77 % neutrophils, respectively, with a low percentage of other non-granulocytic cells.

Extraction of myeloperoxidases from granulocytes. Granulocytes (neutrophils or neutrophil/eosinophil mixtures) were lysed with 0.2 % Triton X-100 in 50 mM potassium phosphate buffer pH 7. Protein was quantified using fluorescamine (Sigma-Aldrich, Oakville, ON, Canada) (147), an amine-reactive fluorophore, as described elsewhere (148). 1×10^6 neutrophils contained 50-70 μg protein. Myeloperoxidases were solubilized by the addition of CETAB to 0.2 %. Extracts were vortexed for 15 s and used to measure myeloperoxidase activity by the bromide-dependent chemiluminescence assay.

Extraction of myeloperoxidase from tumors. Subcutaneous mouse Mutatect tumors, known to be infiltrated with neutrophils (70), were used for measurement of myeloperoxidase by the bromide-dependent chemiluminescence assay. About 25% of each tumor was fixed for histological analysis (see below) and the remainder was used to extract a

cellular fraction (see section 3.5) for the analyses of myeloperoxidase and *hprt* mutation frequency. The tumor fractions were centrifuged and resuspended in 0.2 ml PBS. Cells were lysed by sonication and myeloperoxidase was solubilized by addition of CETAB (0.2 % final) followed by sonication. Protein was quantified using fluorescamine (147), an amine-reactive fluorophore, as described elsewhere (148). Myeloperoxidase activity in the sonicated extracts was measured by the bromide-dependent chemiluminescence assay described below.

Histological analysis of neutrophil infiltration in tumors. Fragments of mouse tumors were transferred to an alcohol-based fixative (Genofix, DNA Genotek Inc., <http://www.DNAGenotek.com>) and embedded in paraffin. Sections of 4 μm were taken and stained with Hemotoxylin and Eosin. The number of neutrophils in the tumor sections was estimated by Dr. Jagdeep Sandhu as follows. A 2-mm grid was drawn on the coverslip over the tumor section. Neutrophils in the corners of each square ($\sim 0.307 \text{ mm}^2$) within the grid were counted using a 40 $\times$ objective. Depending upon tumor size, 5-to-14 squares were chosen at random and scored for neutrophil count. Results were expressed as the mean number of neutrophils per field.

*Analysis of *hprt* mutation frequency.* For complete analyses for the measurement of number of *hprt* mutations in Mutatect tumors see *Chapter 3*.

Extracts of HL-60 and 293T cells. Cultured HL-60 and 293T cells (from ATCC) were lysed in 0.5 % Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 20 mM MOPS, pH 7.2. The lysates were incubated for 30 min on ice, and centrifuged at 12 000 $\times g$ for 20 min at 4°C. The supernatant was used to quantify total protein by fluorescamine (148) and to measure myeloperoxidase activity by the bromide-dependent chemiluminescence assay.

Bromide-dependent chemiluminescence assay for myeloperoxidase activity. Chemiluminescence was measured using a 1251 Luminometer (LKB-Wallac, Finland). Assays were performed in 2 steps at 25°C in a total reaction volume of 1.0 ml in luminometer polystyrene tubes. All manipulations were carried out under subdued lighting. Each sample was assayed in triplicate and at 2 pHs, 5 and 7.5. Initial reaction mixture consisted of 100 μM luminol, 10 mM DTPA (Fe (III) chelator), and 1 mM H_2O_2 in either pH 5 buffer (0.1 M sodium acetate) or pH 7.5 buffer (0.1 M sodium phosphate). Luminol was maintained as stock of 20 mM solution in dimethyl sulfoxide; the presence of dimethyl

sulfoxide ($\leq 2\%$) in the reaction mix had no effect on the chemiluminescence. Step I was started by the addition of 0–4 μg of CETAB-extracted proteins (from neutrophil/eosinophil mixture or tumor extracts) and the tube was immediately transferred into the luminometer. After a 30 s delay, the non-specific chemiluminescence signal was accumulated for 3 min. For step II, a bromide-dependent reaction was initiated by the addition of KBr to 5 mM and another 3 min reading was taken. The myeloperoxidase-specific (bromide-dependent chemiluminescence) reading was obtained by subtracting the non-specific step I reading from the step II reading. One unit of myeloperoxidase activity is defined as the corrected bromide-dependent chemiluminescence 3-min reading.

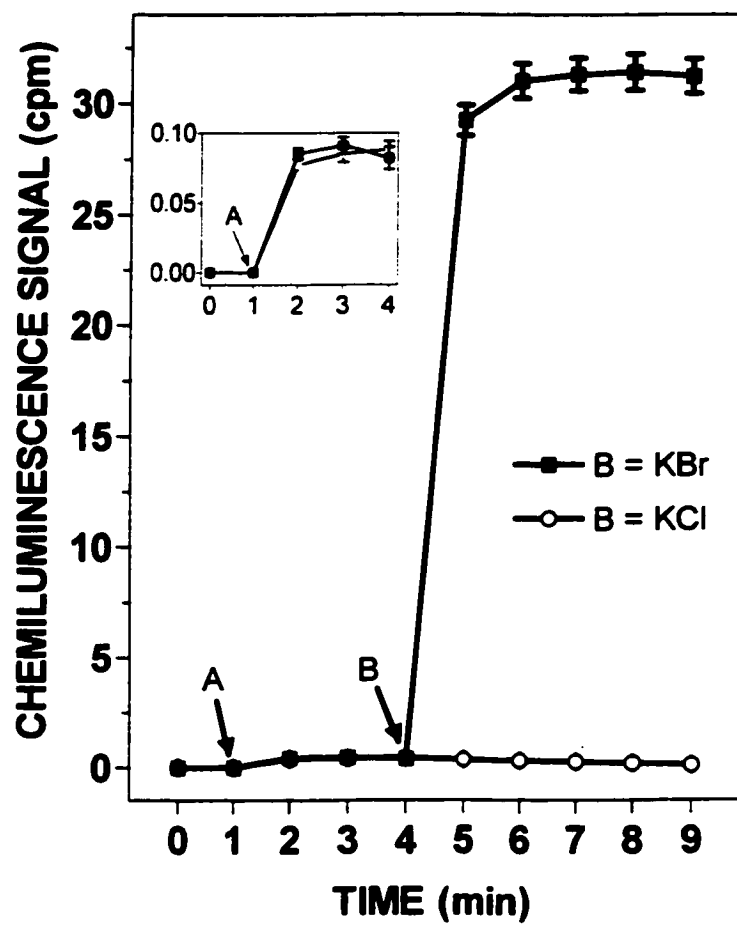
2.6. Results

2.6.1. Neutrophil extracts produce a bromide-dependent chemiluminescence signal

Based upon the unique ability of myeloperoxidase to oxidize bromide in the presence of H_2O_2 , a chemiluminescence assay was designed that would be able to distinguish non-specific signals from myeloperoxidase-specific signals (Fig. 2.1). The specificity of this assay for neutrophil myeloperoxidase was first tested using extracts from human granulocytes containing $>95\%$ neutrophils. The assay consists of 2 steps. The first step uses a solution containing luminol, H_2O_2 , and DTPA at pH 5 to which a myeloperoxidase-containing sample is added. When neutrophil extract was added to this solution, a very low non-specific chemiluminescence was produced (Fig. 2.1, inset). This signal was dependent upon both H_2O_2 and luminol (data not shown). In the second step, bromide is added to the same solution. A significant increase in the chemiluminescence was produced following the addition of bromide (Fig. 2.1). The addition of chloride in place of bromide did not affect the chemiluminescence (Fig. 2.1), which was consistent with an earlier study suggesting that the myeloperoxidase- H_2O_2 -chloride system is almost inactive compared to the myeloperoxidase- H_2O_2 -bromide system at pH 5 (10). CETAB ($\leq 0.01\%$, final concentration) had no effect on the chemiluminescence (not shown). HL-60 cells are malignant cells of the myeloid lineage that contain myeloperoxidase (149). A bromide-dependent signal was also produced by extracts of these cells (data not shown). As a negative control, extracts of 293T cells (an SV40-transformed cell-line of human kidney origin) were tested and, as expected, produced no chemiluminescence signal. We also tested other peroxidase substrates

FIGURE 2.1. The bromide-dependent chemiluminescence assay.

Chemiluminescence signal observed on addition of human neutrophil extract (arrow A; 100 ng protein) and either 5 mM of KBr or KCl (arrow B) to reaction mixtures containing 0.1mM luminol, 1 mM H₂O₂, 10 mM DTPA and 0.1 M acetate buffer (pH 5). See Materials and Methods for other details. The points represent means $\pm$ SD of triplicate experiments. **Inset:** expanded view of step I (non-bromide-dependent) reaction.



(lucigenin and ethidium bromide), but only luminol gave a strong bromide-dependent signal (data not shown). Thus, these results show that neutrophil extracts can catalyze the bromide-dependent chemiluminescence of luminol in the presence of H_2O_2 at pH 5.

2.6.2. Source of the bromide-dependent chemiluminescence signal

Luminol-based chemiluminescence is widely used to assess the myeloperoxidase- H_2O_2 -chloride system and is believed to be a result of the enzymatic oxidation of chloride to hypochlorous acid (HOCl) by myeloperoxidase followed by the chemical reaction of HOCl and H_2O_2 with luminol (140-142, 150). In Fig. 2.2 we demonstrate that addition of hypobromous acid (HOBr) and H_2O_2 to luminol (in the absence of myeloperoxidase) also produced a chemiluminescence signal. This signal was substantially higher than the HOCl-dependent signal at pH 5 (Fig. 2.2). Neither H_2O_2 , HOCl nor HOBr added individually to luminol produced a chemiluminescence signal. We therefore postulate that the bromide-dependent chemiluminescence signal produced by neutrophil extracts in our assay (Fig. 2.1) may result from the reaction of myeloperoxidase-generated HOBr and H_2O_2 with luminol.

2.6.3. Use of the bromide-dependent assay to quantify human neutrophils

We next addressed the question as to whether the assay could be useful in estimating the number of neutrophils. Extracts of pure human neutrophils were used for this analysis. A linear correlation was seen between the bromide-dependent chemiluminescence and the amount of CETAB-extracted protein from neutrophils, up to 0.4 μg per assay (Fig. 2.3). For the linear portion of the curve, the following relationship was obtained:

$$B = 910 x \quad (\text{for } 0.005 \leq x \leq 0.45) \quad [2.5]$$

where B is in units of myeloperoxidase activity and x is μg of neutrophil protein. We determined that the protein content of neutrophils was about 60 μg per 10^6 cells (or 1 μg of protein per 17,000 cells). Thus, the bromide-dependent assay can detect myeloperoxidase corresponding to less than 100 human neutrophils.

FIGURE 2.2. Source of bromide-dependent assay.

Luminol-based chemiluminescence observed on addition of either 10 mM of HOCl or HOBr to reaction mixtures containing 0.1 M acetate buffer (pH 5), 0.1 mM luminol with or without 0.5 mM H₂O₂. A 1-min reading was taken 30 s after addition of HOCl or HOBr. Points represent means $\pm$ SD of triplicate experiments.

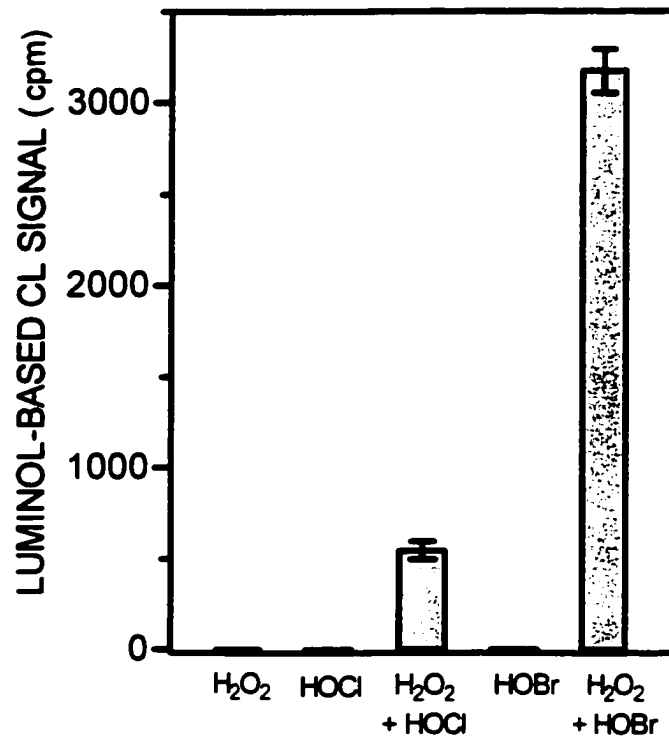
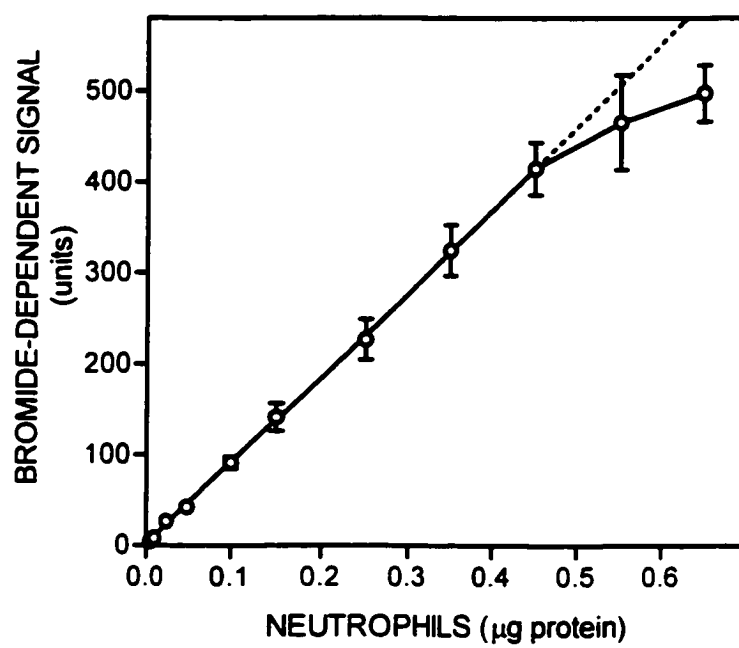


FIGURE 2.3. Neutrophil-derived bromide-dependent chemiluminescence signal.
Varying amounts of a human neutrophil extract were added to a standard bromide-dependent assay. The points represent means $\pm$ SD of three independent experiments.



2.6.4. Specificity of the bromide-dependent assay for myeloperoxidases

A major problem of most myeloperoxidase assays is their inability to discriminate between *myeloperoxidase* and other peroxidases and heme-containing proteins (143). These proteins can catalyze the oxidation of a variety of substrates by H_2O_2 . To determine whether a non-specific peroxidase or a hemoprotein is capable of producing a bromide-dependent signal, we tested horseradish peroxidase and hemoglobin, since these proteins do not catalyze the oxidation of halides to hypohalous acids. Although both horseradish peroxidase and hemoglobin produced non-specific chemiluminescence signals in step I of the assay, no bromide-dependent signals was seen on addition of KBr in step II of the assay (Table 2.1). This indicated that our bromide-dependent assay could differentiate between myeloperoxidase-specific and non-specific reactions. To examine whether horseradish peroxidase or hemoglobin might *interfere* with the bromide-dependent signals produced by myeloperoxidase, assays were carried out on human neutrophil extracts to which horseradish peroxidase or hemoglobin was added (Table 2.1). The bromide-dependent signal of neutrophil extracts was unaffected by the presence of either horseradish peroxidase or hemoglobin. We conclude from these experiments that moderate levels of non-specific peroxidases or heme-containing proteins do not interfere with the bromide-dependent assay since only myeloperoxidases catalyze the oxidation of halides.

2.6.5. Ability of the assay to distinguish between eosinophil peroxidase and myeloperoxidase

Both eosinophil peroxidase and neutrophil myeloperoxidase can catalyze the oxidation of halides in the presence of H_2O_2 and it would be desirable to distinguish the two. To address this, we first tried to distinguish neutrophil myeloperoxidase and eosinophil peroxidase signals using ATA, an inhibitor that has been shown to differentially inhibit the activities of the two enzymes in spectrophotometric assays. In tetramethylbenzidine- and guaiacol-based assays, it has been reported that 0.5 mM ATA inhibits 50 % of eosinophil peroxidase activity while inhibiting only 5 % of neutrophil myeloperoxidase activity (138), an observation confirmed by us (data not shown). However, in our bromide-dependent assay, 35 ± 2 % inhibition of a myeloperoxidase-induced signal was seen. This was attributed to an effect of ATA on the reaction of $HOBr/H_2O_2$ with luminol since 31 ± 2 %

TABLE 2.1: Non-specific and bromide-dependent chemiluminescence of luminol emitted by extracts containing neutrophils, horseradish peroxidase, and/or hemoglobin

Protein extracts ^b	Luminol-based chemiluminescence signal (counts per 3 min) ^a	
	Non-specific	Bromide-dependent
Neutrophils	9.0 ± 0.9	925 ± 22
Horseradish peroxidase	129 ± 17	0.7 ± 7.4
Hemoglobin	53 ± 9	0.1 ± 5.3
Neutrophils + Horseradish peroxidase	138 ± 4	930 ± 57
Neutrophils + Hemoglobin	54 ± 12	916 ± 15

^a Reactions were carried out in the presence of H₂O₂ at pH 5 as described in section 2.5. The values represent mean ± SD of triplicate experiments.

^b Contained 1 µg neutrophil proteins, 2.5 ng horseradish peroxidase and/or 2 µg hemoglobin.

inhibition was seen in this protein-free reaction and 32 ± 1 % inhibition was seen in HL-60-induced bromide-dependent chemiluminescence. HL-60 cells contain myeloperoxidase but no eosinophil peroxidase (149). These results indicate that ATA could not be used for our bromide-dependent assay to distinguish between the two.

We tested different pHs as a means to differentiate between eosinophil peroxidase and myeloperoxidase. Based upon measurement of singlet oxygen production, it has been shown earlier that the eosinophil peroxidase is more active than neutrophil myeloperoxidase at neutral pH (146), whereas neutrophil myeloperoxidase is more active at pH 5 (10). We therefore compared reactions at pH 5 and pH 7.5. Since it is very difficult to completely separate neutrophils from eosinophils, it was necessary to use mixtures containing different proportions of the two types of granulocytes as a source of the 2 peroxidases. At pH 5, a linear relationship was seen between the bromide-dependent chemiluminescence signal and the percentage of neutrophils in the neutrophil/eosinophil mixture (total of 150 ng granulocyte protein) (Fig. 2.4A). The equation of the line was

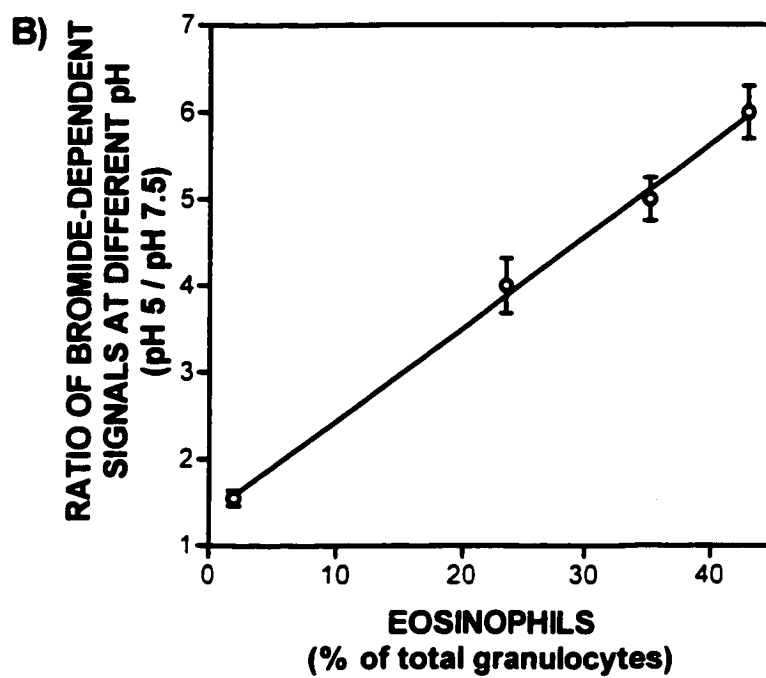
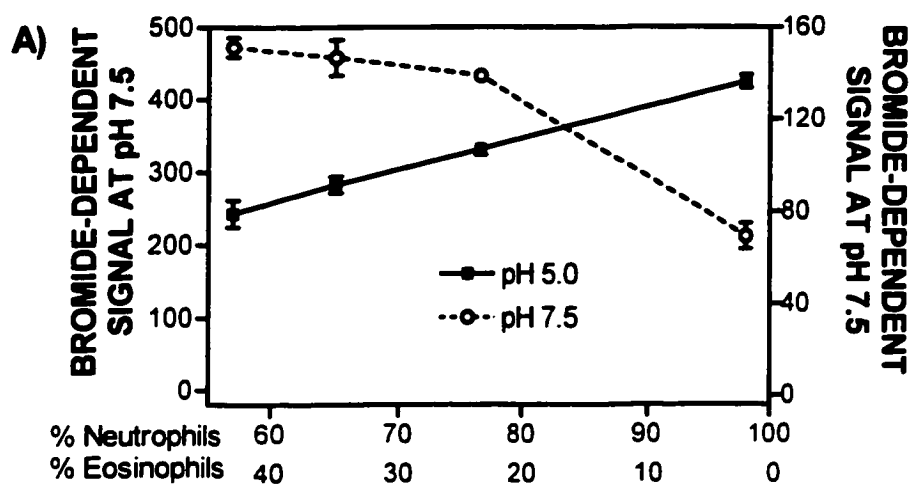
$$B = 1.4a + 0.6 \quad [2.6]$$

where B is the bromide-dependent signal for 150 ng total granulocyte protein, and a is the percentage of neutrophils and $100-a$ is the percentage of eosinophils. Assuming that the extrapolation is linear, a bromide-dependent signal of 0.6 would be expected for 100 % eosinophils. This suggests that eosinophils contribute very little to the bromide-dependent signal at pH 5. At pH 7.5, a direct non-linear relationship was seen between bromide-dependent signal and the percentage of eosinophils in neutrophil/eosinophil mixture (Fig. 2.4A), suggesting that both myeloperoxidase and eosinophil peroxidase are detected at this pH, with a stronger signal contributed by eosinophils. The simplest way to estimate the percentage of eosinophils in mixed granulocyte extracts is to plot of the ratio of the bromide-dependent signals at the 2 pHs (Fig. 2.4B). The linear relationship seen was fitted by the equation

$$E = 9.4r + 13 \quad [2.7]$$

FIGURE 2.4. Bromide-dependent assay to distinguish between myeloperoxidase and eosinophil peroxidase.

A) Bromide-dependent signal generated using extracts containing the indicated ratios of human neutrophils and eosinophils. Assays contained 150 ng of protein and were carried out at pH 5 or pH 7.5. **B)** Ratio of the pH 7.5/pH 5 Br-CL signals as a function of percentage eosinophils in the neutrophil/eosinophil mixtures. Points represent means $\pm$ SD of triplicate experiments.



where E is the percentage of eosinophils and r is the ratio of pH 7.5 to pH 5 signal. This equation was used to estimate the percentage of eosinophils in tumor-infiltrating granulocytes (see below).

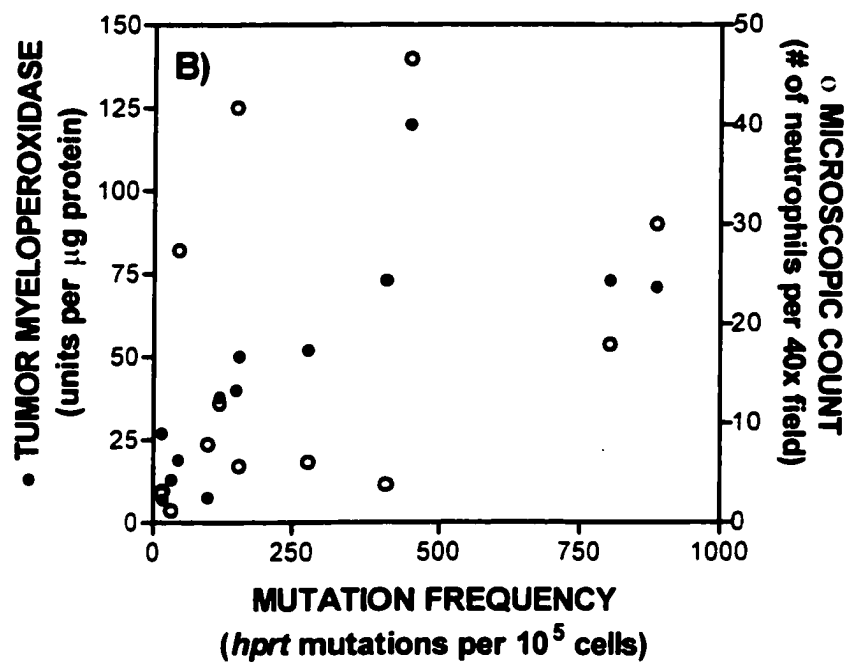
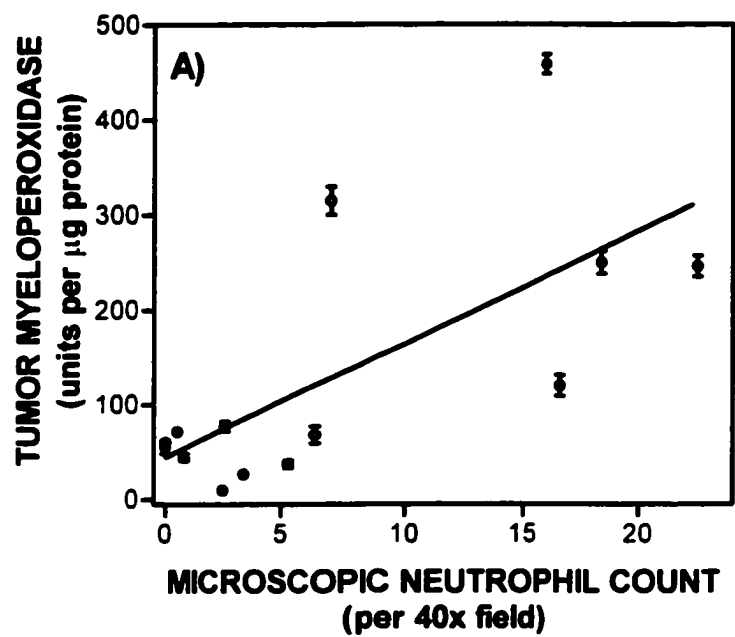
2.6.6. Quantification of neutrophils in Mutatect tumors using the bromide-dependent assay

To test the ability of our bromide-dependent assay to estimate neutrophil content *in vivo*, 14 Mutatect tumors, known to have variable numbers of infiltrating neutrophils (70), were analyzed. Myeloperoxidase was isolated from homogenates of these tumors and quantified using the bromide-dependent assay at pH 5. The activity was compared with a semi-quantitative estimate of neutrophils obtained by microscopically counting of neutrophils in hemotoxylin and eosin-stained histological sections of the tumors (Fig. 2.5A). A statistically significant positive correlation was seen between the semi-quantitative count of neutrophils and the quantitative measurement of myeloperoxidase activity ($r = 0.69$, $p < 0.01$). In an independent experiment, we also compared the neutrophil level measured by myeloperoxidase assay and by microscopic counting with the *hprt* mutation frequency in 13 Mutatect tumors. As shown in Fig 2.5B, the correlation between mutations and neutrophil content was much stronger when the myeloperoxidase assay was used ($r = 0.89$, $p < 0.0001$) than when the microscopic counts were used ($r = 0.60$, $p < 0.03$).

The bromide-dependent myeloperoxidase assay was also carried out at neutral pH. By using the ratio of the bromide-dependent signals at pH 5 to the signal at pH 7.5 (as in Fig. 2.4B), an estimate could be obtained for the number of infiltrating eosinophils. In all tumors, this was very low (0 - 1.5 % of neutrophils), consistent with the observation that the histological sections showed few if any eosinophils (data not shown).

FIGURE 2.5. Utility of the bromide-dependent myeloperoxidase assay.

A) Correlation between mouse tumor myeloperoxidase activity and microscopic counts of neutrophils in histological sections (as described under Materials and Methods). Spearman non-parametric correlation: $r = 0.69$, $P = 0.01$, $n = 14$. **B)** Comparison of neutrophil content measured by myeloperoxidase assay (fill symbols) and by microscopic counting (open symbol) with the *hprt* mutation frequency. Spearman non-parametric correlation: $r = 0.89$, $P = 0.0001$, $n = 13$ for myeloperoxidase assay and $r = 0.60$, $P = 0.03$, $n = 13$ for microscopic counting. The microscopic counting was carried out by Jagdeep Sandhu.



2.7. Discussion

A quantitative method was necessary to reliably measure neutrophil infiltration in Mutatect tumors. Quantification of neutrophils was useful for comparing the levels with other tumor parameters, such as *hprt* mutation frequency (*chapter 3, 4*) and protein nitrotyrosine (*chapter 5*). Earlier studies from our laboratory have relied upon microscopic examination of histological tumor sections for the measurement of neutrophils. Although this type of examination is important in assessing tissue distribution and semi-quantitative estimates of infiltrating neutrophils, quantitative studies are best carried out by biochemical assays of myeloperoxidase, a neutrophil-specific marker present in the azurophilic granules. There are two biochemical methods that are most frequently used for the quantification of myeloperoxidase activity, viz., spectrophotometric and chemiluminescence assays. Spectrophotometric assays commonly use substrates such as tetramethyl benzidine, guaiacol or o-dianisidine at acidic pH (*137, 139, 150*). However, these assays are susceptible to interference by other peroxidases and by certain hemoproteins (*143*). Chemiluminescence assays are based upon the oxidation of luminol by myeloperoxidase- H_2O_2 -halide system. However, these assays are also susceptible to interference by non-specific peroxidases and hemoproteins (*151*) (Table 2.1). In addition, luminol can be oxidized by superoxide ($\text{O}_2^{\cdot-}$) generated by neutrophils in myeloperoxidase-independent reactions (*139, 151*). Therefore, it is difficult to separate the non-specific signals from the myeloperoxidase-specific signals in luminol-based chemiluminescence assays. Furthermore, all published luminol-based assays are carried out at neutral pH (*139, 141, 142*), where the myeloperoxidase- H_2O_2 -halide system is less active than at acidic pH (*10*) and also the eosinophil peroxidase- H_2O_2 -halide system is more active (*146*). In this chapter, a myeloperoxidase assay was described in order to quantitatively and specifically measure neutrophil levels in Mutatect tumors.

The specificity of our bromide-dependent assay is based upon the oxidation of bromide to HOBr, a reaction that is catalyzed by myeloperoxidase and eosinophil peroxidase but not other peroxidases or hemoproteins (see equation 2.1). This oxidation leads to the generation of $^1\text{O}_2$ (see equation 2.4). The combination of HOBr, H_2O_2 and luminol at pH 5 in the absence of myeloperoxidase is sufficient to generate a chemiluminescence signal; a similar but weaker signal was generated by HOCl, H_2O_2 and luminol. It was earlier postulated that myeloperoxidase- H_2O_2 - and chloride could generate HOCl which in turn

could react with luminol and H_2O_2 to produce chemiluminescence (see equation 2.3); the reaction was unaffected by D_2O , suggesting that $^1\text{O}_2$ was not involved (150). We postulate a similar reaction for our bromide-dependent assay, but have not ruled out the possibility that $^1\text{O}_2$ or some other oxidant is involved. The specificity of our assay was further addressed by demonstrating that neither a hemoprotein (hemoglobin) nor a non-specific peroxidase (horseradish peroxidase) could produce a bromide-dependent signal or interfere with an myeloperoxidase measurement. It thus appears that our bromide-dependent assay is useful in distinguishing myeloperoxidase and eosinophil peroxidase from other peroxidases.

To distinguish between the myeloperoxidase and eosinophil peroxidase, we took advantage of the different pH characteristics of the myeloperoxidase- H_2O_2 -halide and the eosinophil peroxidase- H_2O_2 -halide systems (131, 146). The assay was carried out at pH 5 and pH 7.5 on extracts of neutrophil-rich and eosinophil-rich mixtures. At pH 5, the bromide-dependent signal was generated mainly by myeloperoxidase and very little by eosinophil peroxidase. At pH 7.5, both neutrophil and eosinophil extracts produced chemiluminescence, where the latter produced a stronger bromide-dependent signal on a per cell basis. These experiments suggest that our assay is specific for myeloperoxidase if carried out at pH 5. An estimate of the relative number of neutrophils and eosinophils in a preparation can be obtained from the ratio of pH 7.5 to pH 5 signals.

As a demonstration of the utility of our assay, myeloperoxidase levels were measured to estimate neutrophil infiltration in Mutatect tumors, known to be variably infiltrated with neutrophils (70). Myeloperoxidase activity was compared to microscopic counts of neutrophils in histological sections of the tumors. These semi-quantitative counts allowed us to demonstrate a statistically significant correlation between myeloperoxidase activity and neutrophil number. The tumors were also analyzed for *hprt* mutation frequency. As found earlier, a correlation between microscopically-counted neutrophils and mutation frequency was observed. However, neutrophils measured by the myeloperoxidase assay showed a much stronger correlation between neutrophils and mutation frequency. This may be due to the fact that the tumor fractions used for the analysis of myeloperoxidase were the same as the ones used for the analysis of *hprt* mutation frequency. The neutrophil concentration in these fractions was homogenous compared to the tumor sections used for the histological analysis, which had heterogeneous neutrophil infiltration. Thus, neutrophils levels measured

by the quantitative biochemical assay may be more reliable in predicting the *hprt* mutation frequency in Mutatect tumors than the levels measured by the semi-quantitative histological counts.

2.8. Significance

In the previous chapter, construction of interleukin-8-secreting Mutatect tumors was described. The level of neutrophil infiltration, as well as *hprt* mutation frequency, is expected to be high in these tumors. The myeloperoxidase assay described in this chapter was significantly useful in quantitatively measuring the neutrophil content and comparing with the mutation frequency (see *chapter 3, 4*) and with other parameters (see *chapter 5*) in these tumors. This assay was shown to be more reliable than the microscopic neutrophil counts in correlating with mutation frequency. In addition, the assay is less time-consuming than microscopic counting. Since interleukin-8 is also an angiogenic factor, some tumors may have high number of red blood cells as a result of high number of blood vessels. In a conventional myeloperoxidase assay, the hemoglobin from these blood cells could interfere with the assay and lead to misinterpretation of the results. However, the ability of our bromide-dependent assay to distinguish between non-specific (e.g., by hemoglobin and tissue peroxidases) and myeloperoxidase-specific signals should allow accurate measurement of neutrophils in the tumors.

The bromide-dependent assay should also have wider application in examining neutrophil infiltration associated with other conditions. The level of neutrophils in normal tissues is very low but can increase markedly in some inflammatory diseases and during ischemia/reperfusion injury. While histological examination of tissues is important to characterize the nature of the pathology, quantitative estimates of neutrophil infiltration are most reliably done using myeloperoxidase measurements. Recently, our assay was useful in measuring neutrophil infiltration to estimate the severity of acute pancreatitis by an independent group (152). In addition, our ability to distinguish between myeloperoxidase and eosinophil peroxidase was utilized by another independent group to develop an eosinophil peroxidase-specific bromide dependent assay (153). Thus, besides its usefulness in measuring neutrophils in Mutatect tumors, our bromide-dependent assay has many other significant applications.

2.9. Conclusions

A quantitative method to measure neutrophil infiltration in Mutatect tumors was developed. The method is based upon the enzymatic assay for myeloperoxidase, a neutrophil-specific marker. Unlike previously reported assays, the assay described here was capable of differentiating between non-specific and myeloperoxidase-specific signals. The assay was able to quantify neutrophil infiltration in Mutatect tumors and was shown to be more reliable than the microscopic neutrophil counts in predicting other tumors parameters.

Chapter 3. Neutrophil content and genetic instability in interleukin-8-secreting Mutatect tumors

3.1. Chapter Summary

Neutrophils represent a potential source of genotoxic reactive oxygen and nitrogen species in the tumor microenvironment. Mutatect tumors are known to be variably infiltrated with neutrophils. Neutrophil number has been shown to correlate with mutations arising *in vivo* in Mutatect cells at the *hprt* locus, a marker of genetic damage. To demonstrate the mutagenic capability of neutrophils, our laboratory previously attempted to increase the levels of neutrophils in the tumors by directly injecting interleukin-8 protein, a chemokine of neutrophils. However, these injections were not able to consistently chemoattract neutrophils. To provide a consistent source of interleukin-8 in tumors, interleukin-8-secreting Mutatect cell lines were developed (*chapter 1*). In *chapter 2*, a quantitative assay for myeloperoxidase, a neutrophil-specific marker, was also developed for measuring neutrophil infiltration in Mutatect tumors. In the present chapter, the effect of interleukin-8 expression on neutrophil infiltration (as measured by myeloperoxidase activity) and on *hprt* mutation frequency is described. In addition, stability of interleukin-8 gene *in vivo* and *in vitro* was also examined. In a series involving 45 animals, interleukin-8-expressing lines produced tumors with a consistently higher neutrophil content than the control line. Analysis of the 45 tumors revealed that the neutrophil level again strongly correlated with *hprt* mutation frequency ($P < 0.0001$, $r = 0.88$). Administration of tetracycline was effective in lowering the neutrophil content of low interleukin-8-expressing tumors but not high. Although the interleukin-8 transgene was stable in all lines *in vitro*, high interleukin-8-expressing lines completely lost the transgene *in vivo* while low interleukin-8-expressing lines showed no evidence of transgene instability. These results provide further evidence, based upon the study of an endogenous gene (*hprt*) and an interleukin-8 transgene, that neutrophils may contribute to genetic instability in tumors.

3.2. Introduction

Inflammatory cells such as neutrophils and macrophages are frequently found to infiltrate human tumors, including pulmonary adenocarcinoma, glioma, lymphoma, gastric carcinoma, melanoma, breast carcinoma and colorectal and prostate cancers (45-59). In some cases, the level of infiltration with inflammatory cells has been correlated with parameters associated with tumor progression. Inflammatory cells are capable of releasing potentially mutagenic compounds, such as reactive nitrogen oxide species (see *General Introduction*). Since accumulation of mutations (genetic instability) in tumors is believed to be the driving force in tumor progression, we and others have proposed that the mutagenic species generated by tumor-infiltrating inflammatory cells are in some measure responsible for the accumulation of mutations associated with tumour progression (65-68).

Our laboratory previously established the Mutatect tumor model to study the contribution of tumor-infiltrating inflammatory cell-derived mutagenic species to genetic instability *in vivo* (see *General Introduction*). This model permits detection of mutations at the hypoxanthine phosphoribosyltransferase (*hprt*) locus in cells recovered from subcutaneous tumors in syngeneic C57BL/6 mice (66, 154). The *hprt* gene is used as surrogate marker of mutations occurring elsewhere in the genome. The seminal observation arising from these early studies was a 1.5-to-5.5-fold higher *hprt* mutation frequency *in vivo* than *in vitro* (mean of 4-fold) (66, 107). This led us to examine the possibility that mutagenic factors are present in the tumor microenvironment. Histological examination of the Mutatect tumors showed frequent infiltration of inflammatory cells, mainly neutrophils. Microscopic counting of these neutrophils demonstrated a strong correlation between the number of neutrophils and *hprt* mutation frequency (70). Further examination showed that the neutrophils stained positively for inducible nitric oxide synthase (NOS), a source of reactive nitrogen oxide species; in addition, there was a correlation between NOS activity and the *hprt* mutation frequency. Furthermore, the tumor cells stained positively for nitrotyrosine (an immunohistochemical marker of RNOS) (70) (see *chapter 5*). To demonstrate that the neutrophils are in fact capable of inducing mutations, our laboratory attempted to increase the levels of neutrophils in the tumors. Interleukin-8 protein, a chemokine of neutrophils, was directly injected into established Mutatect tumors. This led to a significant increase in the neutrophil infiltration but only in 22% of the interleukin-8-

injected tumors (i.e., 8 out of 36) (70). This was likely due to inconsistencies with interleukin-8 injections. In order to consistently chemoattract a high number of neutrophils, I developed a series of Mutatect cell lines that secrete interleukin-8 from a constitutive or a tetracycline-regulatable promoter (*chapter 1*). These include MIL-4, TM-3, TM-7, TM-28 and TM-34. The tumorigenicity of these cell lines was also described. In *chapter 2*, a quantitative assay for myeloperoxidase, a neutrophil-specific marker, was also developed for measuring neutrophil infiltration in Mutatect tumors. In the present chapter, the effect of interleukin-8 expression on neutrophil infiltration (as measured by myeloperoxidase activity) and on *hprt* mutation frequency is described. In addition, in *chapter 1*, it was observed that neomycin resistance was “lost” in MIL-4 tumors. Since the neomycin gene is linked to the interleukin-8 gene in these cells, the stability of interleukin-8 gene in all cell lines growing *in vivo* and *in vitro* was also examined.

3.3. Hypotheses

A large number of neutrophils could be consistently chemoattracted in Mutatect tumors if the tumors themselves produce interleukin-8. The chemoattracted neutrophils would be capable of inducing a higher *hprt* mutation frequency in the tumor cells.

3.4. Initial objectives and expected results

The overall objective was to identify interleukin-8-secreting Mutatect cell line(s) that consistently produce tumors with high number of neutrophils and high number of *hprt* mutations. The long-term objective was to use such tumors to study the effect of dietary antioxidants on genetic instability. The followings were the initial objectives I set out to examine in this chapter:

1. *To quantify myeloperoxidase activity, as a measure of neutrophil content, in interleukin-8-secreting Mutatect tumors.* It was expected that a higher number of neutrophils would be recruited in interleukin-8-secreting tumors than non-secreting tumors. In addition, the number of neutrophil would depend upon the level of interleukin-8 secreted by the tumor cells.
2. *To examine interleukin-8 tumors by histology in order to confirm neutrophil content.* Interleukin-8-secreting tumors were expected to have high neutrophil infiltration.

3. *To examine whether neutrophil content was affected in MIL-4/MC17-51 mixed tumors and tetracycline-receiving TM tumors.* It was expected that neutrophil levels would be lower in both types of the tumors, mixed and tetracycline-receiving.
4. *To measure *hprt* mutation frequency in interleukin-8 tumors.* It was expected that interleukin-8-secreting tumors would have a higher mutation frequency than non-secreting tumors. In addition, the level of mutation frequency would depend upon the level of interleukin-8 secreted by the tumor cells.
5. *To establish if a correlation existed between neutrophil content and *hprt* mutation frequency.* A direct correlation between the two parameters has been previously observed in non-interleukin-8-secreting tumors and was expected to be also true for interleukin-8-secreting tumors.
6. *To measure the stability of interleukin-8 expression in tumors.* Interleukin-8 expression was expected to be little affected throughout tumor development.

3.5. Materials and Methods

Reagents. Fluorescamine, diaminobenzoic acid, hypoxanthine, aminopterin, thymidine, 6-thioguanine (6-TG) and tetracycline hydrochloride for cell culture were from Sigma-Aldrich (Oakville, Canada). G-418 was from GIBCO-BRL (Burlington, Canada) and rabbit anti-myeloperoxidase polyclonal antibody was from Lab Vision Corporation (Fremont, CA). Tetracycline hydrochloride administered to animals in the drinking water was diluted from a flavoured suspension for oral use (Novo-Tetra, Novopharm, Toronto, Canada).

Mutatect cell lines and tumor analyses. All cell lines were maintained in Dulbecco's modified Eagle's medium plus 10 % fetal calf serum (GIBCO- BRL, Burlington, Canada) in a humidified atmosphere of 5 % CO₂ at 37°C. Mutatect cell lines used in this chapter include MC17-51, MT-6 and interleukin-8-secreting MIL-4, TM-3, TM-7, TM-28 and TM-34. Details and development of these cell-lines are described in *section 1.5*. Any spontaneously-arising background *hprt* mutants were eliminated by "HAT-cleaning" to obtain a ~100% *hprt*⁺ population as follows: cells were grown for 7 days in HAT medium (100 µM hypoxanthine, 400 nM aminopterin, 15 µM thymidine) and 2 days in HT medium (no aminopterin). These cells were used to produce subcutaneous tumors in C57BL/6 mice

as described in *section 1.5*. For *in vitro* controls, the same cells and cell-mixtures were also grown in culture for the entire period of *in vivo*-tumor growth, subculturing twice weekly. Where indicated, 0.4 mM tetracycline was added to the drinking water of the animals; the tetracycline water was protected from light and changed every 2-3 days. When the largest diameter of non-interleukin-8-producing control tumors reached 1 cm (i.e., by ~3 weeks), all tumor-bearing mice were euthanised and their tumors harvested. About 25 % of each tumor was used for histological analysis and for isolation of DNA for PCR analysis of the interleukin-8 transgene. The remainder of the tumor was lightly minced in 5 ml of Dulbecco's modified Eagle's medium plus 10% fetal calf serum and mechanically dispersed by passage through a 3 cc-syringe. The suspension was incubated in a 15 ml tube at 0°C for 3-5 min and the particulate (stromal fraction) was allowed to settle. The supernatant (cellular fraction) was separated and used for the measurement of myeloperoxidase activity and for *ex vivo* growth of tumor cells⁴. After 2-3 days of *ex vivo* growth, the cells were analyzed for neomycin resistance, number of *hprt* mutations, secretion of interleukin-8 protein and presence of the interleukin-8 transgene. All experiments using animals were carried out at the Animal Care & Veterinary Service of the University of Ottawa in accordance with guidelines of the Canadian Council on Animal Care.

Myeloperoxidase analyses. The cellular fraction from each tumor was centrifuged and resuspended in 0.2 ml PBS. Cells were lysed by sonication and myeloperoxidase was solubilized by addition of cetyltrimethylammonium bromide (0.2 % final; Sigma-Aldrich) followed by sonication. Protein was quantified using fluorescamine (147), an amine-reactive fluorophore, as described elsewhere (148). Myeloperoxidase activity in the sonicated extracts was measured by the bromide-dependent chemiluminescence assay described in *chapter 2* (155). One unit of myeloperoxidase activity is defined as the corrected 3-min-bromide-dependent reading. Each sample was assayed in triplicate.

Analysis of neomycin resistance. The percentage of neomycin resistant cells were measured in Mutatect cells growing *ex vivo* and *in vitro*. With the exception of the parental MC17-51 cells, all Mutatect cell lines (MIL-4, MT-6, TM-3, TM-7, TM28 and TM-34) had been stably transfected with a gene for neomycin resistance (aminoglycoside

⁴ No difference in myeloperoxidase activity per amount of protein was observed in the tumors between cellular and stromal fractions.

phosphotransferase) during their derivation (see *section 1.5* for details). This characteristic was utilized to measure the percentage of Mutatect cells in *ex vivo*-growing tumors and the percentage of MIL-4 cells in the MIL-4/MC17-51 mixed tumor population. For estimating the percentage of neomycin resistant cells, 200 viable cells were plated in triplicate on 6-cm dishes in the absence or presence of 500 µg/ml G-418 (a neomycin analog). After 8 days of incubation, the colonies formed were counted and the percentage of neomycin resistant cells as a fraction of the total cells plated was calculated. About 90-95% of the *ex vivo*-growing tumor cells were Mutatect cells, while the remainder were host cells.

Analysis of hprt mutation frequency. The frequency of *hprt* mutants was measured using 6-thioguanine in Mutatect cells growing *ex vivo* and *in vitro*. 6-thioguanine is a non-toxic drug that can be catalyzed into a cytotoxic intracellular species by the *hprt* enzyme; hence, only *hprt* mutant cells survive this drug. 1×10^5 viable cells from each tumor were plated in triplicate in 10-cm dishes in medium containing 50 µM of 6-thioguanine. After 12-14 days of incubation, the number of 6-thioguanine-resistant colonies (representing *hprt* mutants) was counted and corrected for plating efficiency. Mutation frequency is expressed as the number of *hprt* mutants per 1×10^5 clonable tumor cells.

Interleukin-8 protein detection by ELISA. Analysis of interleukin-8 in culture medium was carried out using a competitive ELISA with rabbit anti-human interleukin-8 polyclonal antibody (Endogen, Woburn, MA), as described in *section 1.5*.

Interleukin-8 transgene detection by PCR. Mutatect cells recovered from tumors (i.e., growing *ex vivo*) and their *in vitro*-controls were analyzed for the presence of a fragment of the transfected interleukin-8 gene. Genomic DNA was isolated from cultured cells or from total Mutatect tumors as described elsewhere (156). The DNA (100 ng) was analyzed by polymerase chain reaction (PCR) to amplify an interleukin-8-specific fragment using forward primer GAGGCCTATATAAGCAGAGC and the reverse primer AGAGCTGCAGAAATCAGGAA. For MIL-4 cells, a 214 bp fragment from the integrated pcDNA3/IL8 transgene (*section 1.5*) was amplified; this corresponded to 110 bp of the cytomegalovirus promoter of pcDNA3 and 104 bp of the 5' end of interleukin-8 cDNA (i.e., the entire 5' untranslated region and the first 56 nucleotides of the coding region). For all TM clones, a 241 bp fragment from the integrated pTRE-IL8 transgene (*section 1.5*) was amplified; this corresponded to 137 bp of the TRE promoter and 104 bp of the 5' end of

interleukin-8 cDNA. Failure to detect the specific 214 or 241 bp fragment, while detecting a non-specific 670 bp fragment product on an ethidium bromide-stained agarose gel, was taken as evidence of interleukin-8 gene loss.

Statistical Analysis. Non-parametric statistical tests were used unless otherwise indicated. The Mann-Whitney U test was used to compare 2 unpaired groups. Spearman rank correlation was used to determine a correlation between 2 measured variables. A value of $P < 0.05$ was considered to be statistically significant. All P values shown are 2-tailed.

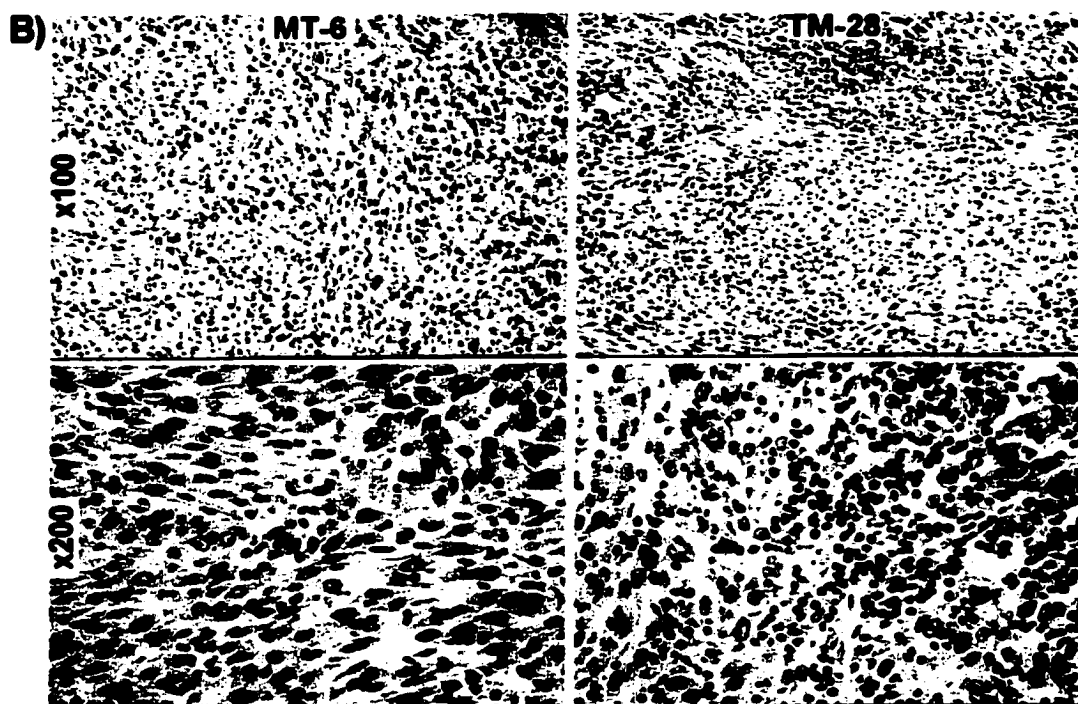
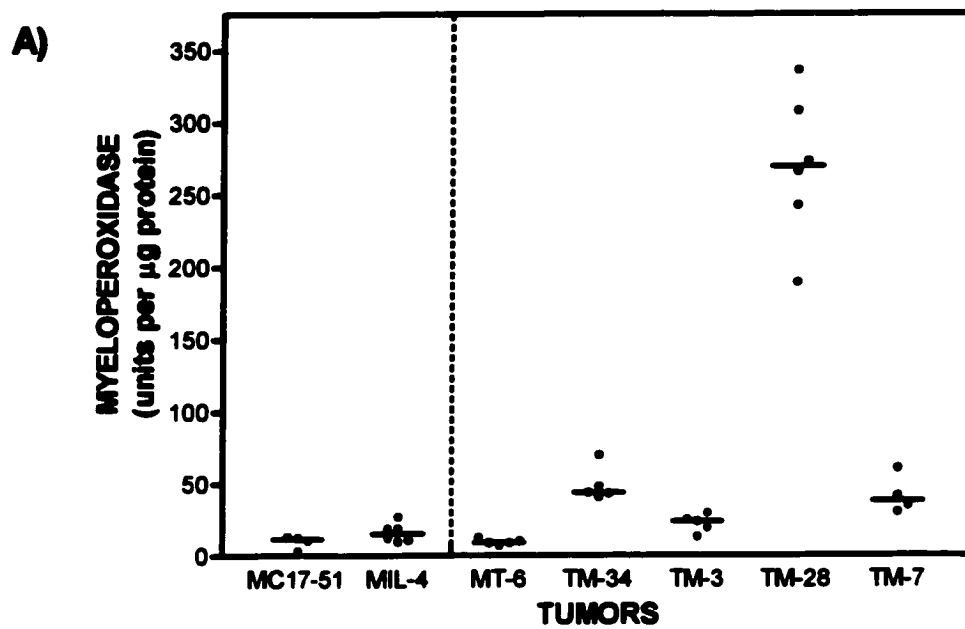
3.6. Results

3.6.1 Neutrophil content in interleukin-8-secreting Mutatect tumors

Interleukin-8 is a potent chemoattractant for neutrophils. In *Chapter 1*, development of several interleukin-8-secreting Mutatect cell-lines and their establishment as subcutaneous tumors was described. To examine whether interleukin-8 secretion was able to successfully increase the number of infiltrating-neutrophils in these tumors, a biochemical assay for myeloperoxidase that is capable of specifically and quantitatively measure neutrophil content (described in *Chapter 2*) was utilized. Myeloperoxidase was measured in tumors formed by cells that secreted high (MIL-4 and TM-7), intermediate (TM-28) or low (TM-3 and TM-34) levels of interleukin-8 and compared with those formed by non-secreting cells (MC17-51 and MT-6). All the tumors formed by interleukin-8-secreting cells had a statistically significantly higher myeloperoxidase than the tumors formed by non-secreting control cells (Fig. 3.1A). This demonstrated that interleukin-8 secretion was able to increase the neutrophil infiltration into tumors. However, the neutrophil level did not correlate with the initial interleukin-8 level secreted by the cell types. Intermediate interleukin-8-secreting TM-28 cells had the highest level of neutrophil content in their tumors, whereas, both high and low interleukin-8-secreting cells (MIL-4, TM-7, TM-3, TM-34) had relatively low levels of neutrophil in their tumors (Fig. 3.1A). Myeloperoxidase activity is a specific marker of neutrophils (see *Chapter 2*). To further confirm that high myeloperoxidase activity in TM-28 tumors was in fact due to high neutrophil infiltration, sections of the tumors were examined by histology (Fig. 3.1B). An anti-myeloperoxidase antibody was used to facilitate detection of the neutrophils. The TM-28 tumors were found to have a heavy infiltration of neutrophils, whereas the non-interleukin-8-secreting MT-6 tumors had low levels of

FIGURE 3.1. Neutrophil content in interleukin-8-secreting Mutatect tumors.

A) Myeloperoxidase levels in Mutatect tumors at day 19. Each point represents a single tumor and the horizontal bar represents the median. Mutatect MC17-51 and MT-6 are control non-interleukin-8 producing tumors, while the others are interleukin-8 producing tumors. **B)** Neutrophil detection in histological sections of MT-6 or TM-28 tumors. The detection of neutrophils (brown staining) was facilitated using an anti-myeloperoxidase antibody and was carried out by Dr. Jagdeep K. Sandhu. Other details are described in Materials and methods



infiltration. Thus, these experiments confirm that interleukin-8 is biologically functional as a chemoattractant for neutrophils in Mutatect tumors.

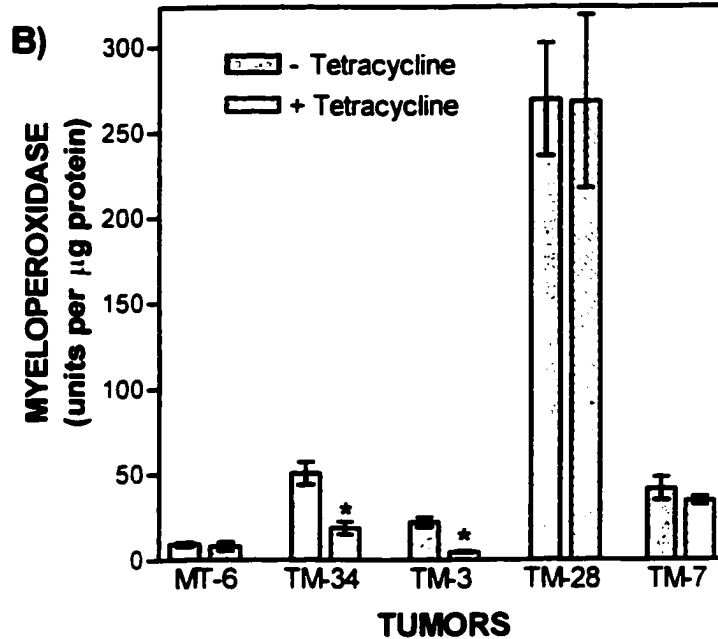
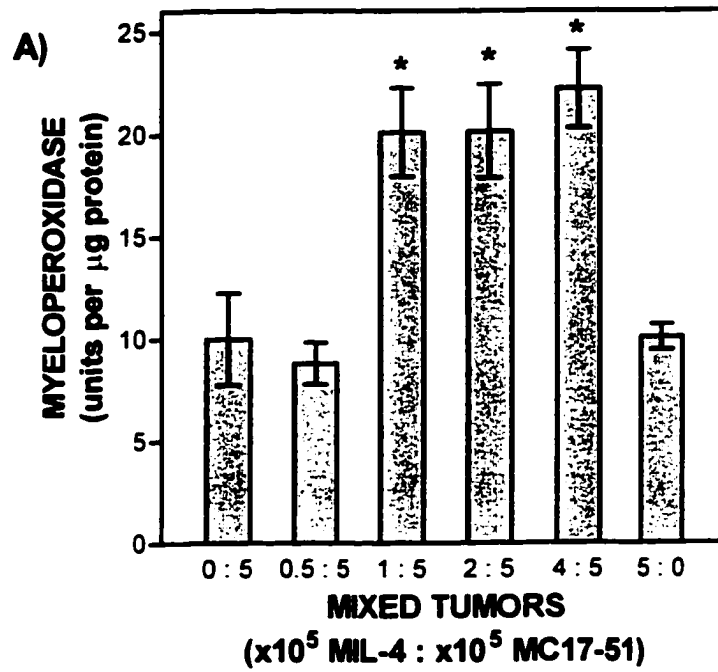
Secretion of interleukin-8 in Mutatect MIL-4 cells is constitutive. In Mutatect TM-7, TM-28, TM-3 and TM-34 cells, it can be regulated by tetracycline (see *Chapter 1*). We therefore attempted to manipulate neutrophil levels in tumors using 2 different methods: (i) by forming tumors with mixtures of MIL-4 and non-interleukin-8-secreting MC17-51 cells, and (ii) by administering tetracycline in the drinking water of mice bearing the tumors of TM cells to suppress interleukin-8 production. For the first method, tumors of the MC17-51 and MIL-4 cell-mixtures of ratios 1:5, 2:5 and 4:5 were analyzed for neutrophil content by myeloperoxidase activity. The tumorigenicity of these mixtures is described in *section 1.6.3*. The neutrophil content was found to be significantly higher in 1:5, 2:5 and 4:5 mixed tumors compared to pure MIL-4 or MC17-51 tumors (Fig. 3.2A). For the second method of manipulation of neutrophil infiltration, mice bearing tumors of TM cells were given tetracycline in their drinking water. Neutrophil levels in tumors formed by cells that initially secreted high (TM-7) or intermediate (TM-28) levels of interleukin-8 were unaffected by tetracycline (Fig. 3.2B). However, tumors formed by cells that secreted low levels of interleukin-8 (TM-3 and TM-34) were clearly responsive to tetracycline. The neutrophil content in these tumors was completely suppressed by tetracycline to the level seen in non-interleukin-8-secreting tumors. Thus, neutrophil infiltration in Mutatect tumors can be regulated by tetracycline *in vivo* in the case of low interleukin-8-secreting, tetracycline-responsive lines.

3.6.2 *Hprt* mutation frequency in interleukin-8-secreting Mutatect tumors

Mutatect cells were developed to permit detection of mutations that arise *in vivo* at the *hprt* gene due to factors in the tumor microenvironment (154); mutations at the *hprt* locus are used as surrogate marker for mutations arising elsewhere in the tumor. Our lab has previously shown that the *hprt* mutation frequency is 4-fold higher in Mutatect cells growing subcutaneously as solid tumors than in the cells growing in culture (154), suggesting that the tumor microenvironment contains mutagenic factors. To investigate the effect of interleukin-8 secretion on mutation frequency, *ex vivo* analysis of *hprt* mutations was carried out in tumors that were formed by high (MIL-4, TM-7), intermediate (TM-28) or low (TM-3,

FIGURE 3.2. Manipulation of neutrophil levels in Mutatect tumors.

A) Myeloperoxidase levels in mixed MIL-4/MC17-51 tumors. B) Myeloperoxidase levels in tetracycline-regulatable Mutatect tumors. Tetracycline (0.4 mM) was added to the drinking water of mice either throughout tumor development (+ tetracycline) or only for the first week (– tetracycline). The "–tetracycline" results have been replotted from Fig. 3.1. Each bar represents mean $\pm$ SEM of 5-to-8 tumors. Other details in Materials and Methods.

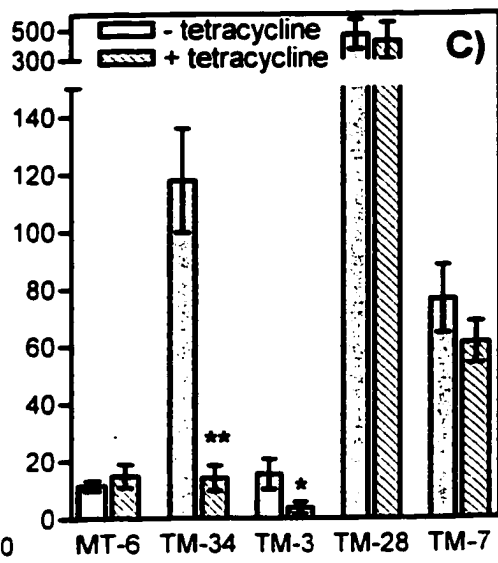
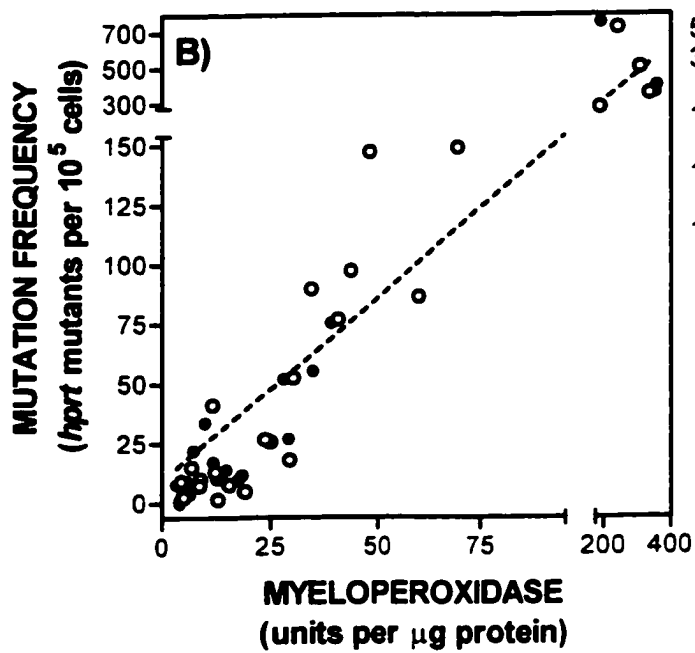
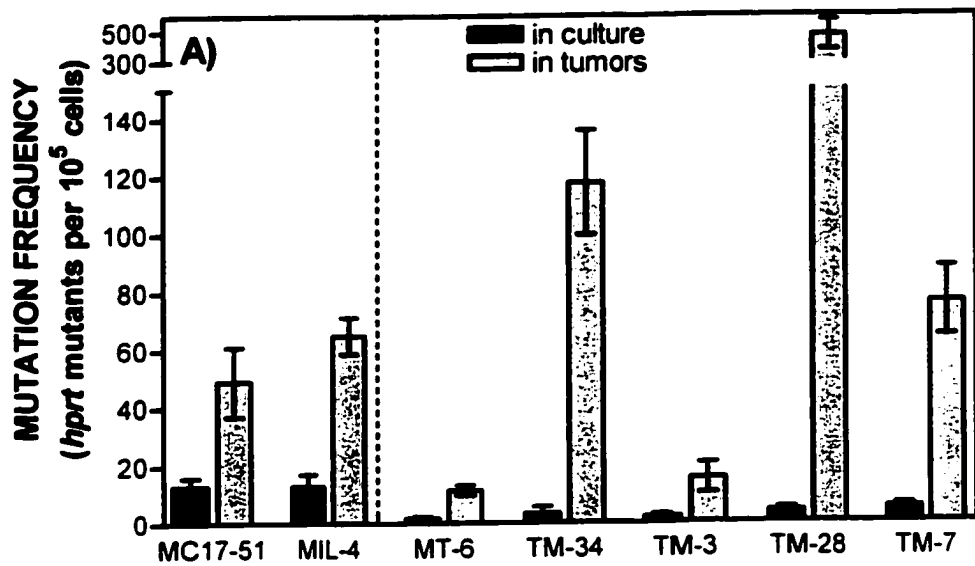


TM-34) interleukin-8-secreting cells and compared with those formed by non-secreting cells (MC17-51 and MT-6). Mutation frequency was also analyzed in the same cell types growing in culture for the entire period of *in vivo*-tumor growth (Fig. 3.3A). In all the cell types examined, the *hprt* mutation frequency was significantly higher in cells growing as subcutaneous tumors than in cells growing in culture. This confirmed our previous observation that the tumor microenvironment contains mutagenic factors (66) and suggested that interleukin-8 secretion on its own does not affect mutation frequency. When the mutation frequency among all the subcutaneous tumors was compared, it was found that the tumors formed by interleukin-8-secreting cells had a statistically significantly higher *hprt* mutation frequency than the tumors formed by non-secreting control cells (Fig. 3.3A). Thus, these results suggest that the tumor microenvironment contain mutagenic factors whose effects are enhanced in interleukin-8-secreting tumors.

The frequency of *hprt* mutations in the tumors (Fig. 3.3A) closely paralleled the measurement of neutrophil content (Fig. 3.1A, 3.2B). Intermediate interleukin-8-secreting TM-28 cells had the highest mutation frequency in their tumors, whereas both high and low interleukin-8-secreting cells (MIL-4, TM-7, TM-3, TM-34) had relatively low mutation frequency in their tumors. The *hprt* mutation frequency has been previously shown to correlate with the number of intratumoral neutrophils (70). To determine whether a similar correlation exists for interleukin-8-secreting cell lines, the 45 tumors shown in Fig. 3.3A and 3.2B were analyzed. A strong statistical correlation between myeloperoxidase and *hprt* mutant frequency was seen ($P < 0.0001$, $r = 0.88$) (Fig. 3.3B). Mutations were also examined under conditions in which neutrophil infiltration had been manipulated, i.e., in mixtures of MIL-4/MC17-51 tumors and in tetracycline-receiving TM tumors. Similar to the effect seen on neutrophil content (see above), the mutation frequency was significantly higher in 1:5, 2:5 and 4:5 mixed tumors compared to pure MIL-4 or MC17-51 tumors (data not shown) and could be inhibited by tetracycline *in vivo* in the case of low interleukin-8-secreting TM-3 and TM-34 cell lines (Fig. 3.3C). A strong correlation between neutrophil content and *hprt* mutation frequency was also seen in these tumors (not shown). Thus, these results further confirm the association between infiltrating-neutrophils and mutation frequency and suggest that the neutrophils are the likely source of the mutagenic factors in the tumor microenvironment.

FIGURE 3.3. *Hprt* mutation frequency in Mutatetect cells *in vitro* and *in vivo*.

A) Comparison of *hprt* mutation frequency in cell recovered from tumors after 3 weeks of growth and in cells growing in culture for an equivalent period. **B)** Correlation between myeloperoxidase levels and *hprt* mutation frequency. Spearman rank correlation: $n = 45$, $P < 0.0001$, $r = 0.88$. Open symbols = –tetracycline; closed symbols = +tetracycline. **C)** Effect of tetracycline on *hprt* mutation frequency. Tetracycline (0.4 mM) was added to the drinking water of mice either throughout tumor development (+ tetracycline) or only for the first week (– tetracycline). Each bar represents mean $\pm$ SEM of 5-to-8 tumors. Other details in Materials and Methods.



3.6.3 Stability of interleukin-8 secretion in Mutatect tumors

Stability of the secretion of interleukin-8 protein was also determined in the *in vitro*- and *ex vivo*-growing cells after >3 weeks of *in vivo* Mutatect tumor-growth. Interleukin-8 level was measured in the culture media of the cells using ELISA and compared with the *initial* level secreted by the cells (see Fig. 1.2 and 1.8B). For *in vitro*-growing cells, the interleukin-8 level was comparable to the initial levels in all the cell-types, suggesting that the secretion of interleukin-8 is stable in culture (not shown). However, for *ex vivo*-growing cells, the interleukin-8 level depended upon the initial level. TM-3 and TM-34 cells, which initially produced low levels of interleukin-8, were found to secrete similar levels of interleukin-8 *ex vivo* (Fig. 3.4A). On the other hand, MIL-4, TM-7 and TM-28 cells, which initially produced high or moderate levels of interleukin-8, were found to secrete very low levels of interleukin-8 *ex vivo*, <20% of the initial levels ($P < 0.0002$ for each cell type; 2-tailed t-test). Thus, when grown as tumors, the interleukin-8 expression is stable only in cells that initially secrete low levels of interleukin-8 but not in cells that secrete moderate-to-high levels; on the other hand, when grown in culture, the expression is stable in all cell types regardless of the interleukin-8 level.

To study the effect of lowering the initial concentration of interleukin-8 on the stability of secretion, tumors formed by mixtures of high interleukin-8-secreting MIL-4 and non-secreting MC17-51 cells were examined. Cells isolated from these tumors were found to secrete a significant amount of interleukin-8 that was comparable to, or even higher than (>100%), the corresponding *in vitro* mixed cultures i.e., not transplanted into animals (Fig. 3.4B). This was in sharp contrast to the very low level of secretion from the cells isolated from *pure* MIL-4 tumors, which corresponded to <20% of the *in vitro* controls (see above). Thus, these results further suggest that the lack of stability of interleukin-8 expression in MIL-4 tumors was likely due to a high initial concentration of interleukin-8.

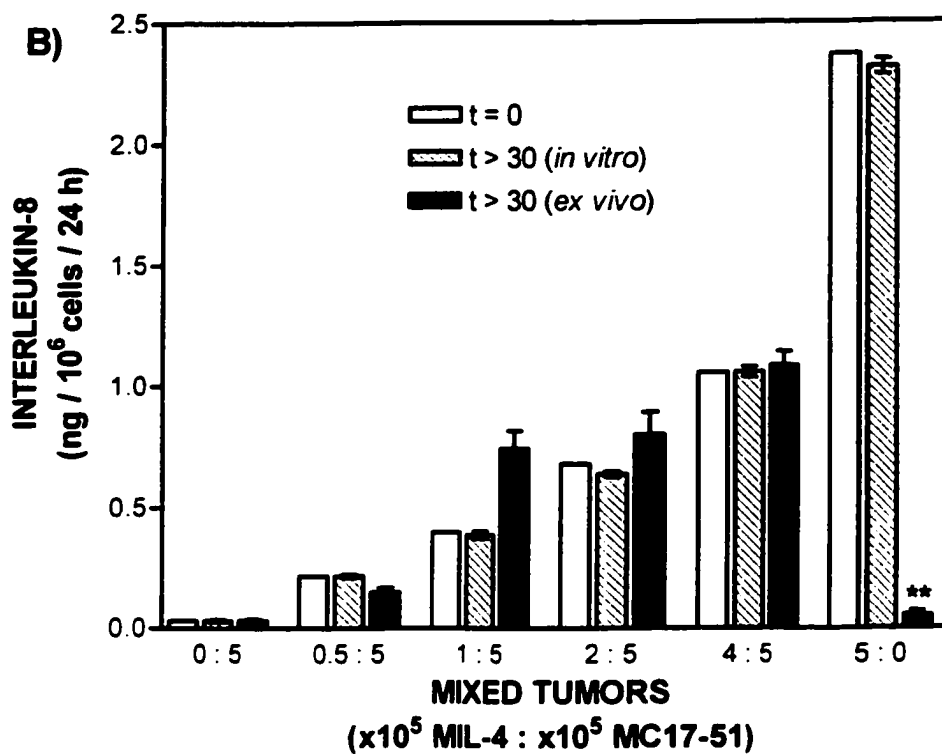
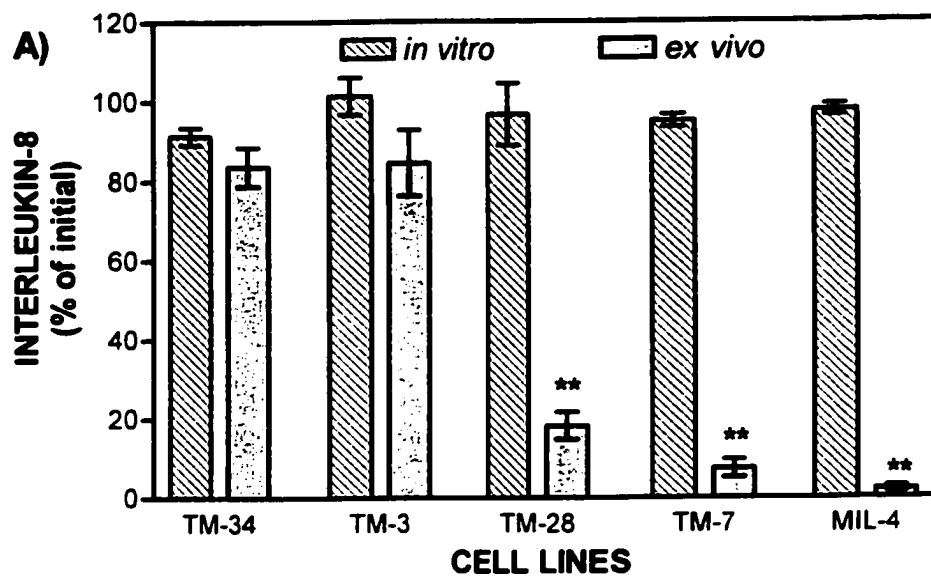
3.6.4 Status of interleukin-8 transgene in Mutatect tumors

Interleukin-8-secreting Mutatect cells were developed by stable transfection of an exogenous transgene containing the cDNA for interleukin-8 (see *Chapter 1*). As shown above, *in vivo* interleukin-8-protein secretion was absent in cells that initially secreted high (MIL-4, TM-28) or moderate (TM-28) levels but not in cells that secreted low (TM-3,

FIGURE 3.4. Comparison of interleukin-8 secretion by cells recovered from Mutatect tumors (*in vivo*) with the cells growing in culture (*in vitro*).

A) Percent of the initial amount of interleukin-8 detected in TM-34, TM-3, TM-28, TM-7 and MIL-4 cells. **B)** Detection of interleukin-8 levels in mixed MIL-4/MC17-51 cultures growing *in vitro* or as tumors and comparison of the level with the initial level ($t = 0$).

Interleukin-8 was measured using ELISA. Each bar represents mean $\pm$ SEM of 5-to-8 tumors. Other details are provided in Materials and methods.



TM-34) levels. We therefore examined whether the lack of interleukin-8-protein expression in the tumors was a result of loss of a functional transgene. The first evidence that suggested that the transgene was non-functional came from the examination of neomycin resistance in *ex vivo*-growing cells (see Fig. 1.5B). In MIL-4 cells, the interleukin-8 gene is linked to the neomycin gene, while in all the TM cells, the genes are likely un-linked since they were transfected separately (see section 1.5). Examination of neomycin resistance showed that MIL-4 tumors were only <5% resistant to neomycin (Fig. 1.5B), while all the TM tumors were >90% resistant (data not shown). Mixed MIL-4/MC17-51 tumors were also found to be resistant to neomycin (Fig 1.5B), which was consistent with the results obtained above for interleukin-8 protein secretion (Fig 3.4B). In fact, a strong correlation between interleukin-8 secretion and neomycin resistance was seen in tumors of pure and mixed MIL-4 (Fig. 3.5). To confirm the absence of the transgene, genomic DNA from MIL-4, TM-7, TM-28 TM-34 and TM-3 tumors was isolated and analyzed by PCR for the presence of an interleukin-8-specific fragment, corresponding to the 5'-end of its cDNA. Little or none of this PCR fragment could be detected in the DNA of the MIL-4, TM-7 or TM-28 tumors, while the fragment was clearly detectable in the DNA of TM-34, TM-3 and all mixed MIL-4/MC17-51 tumors (Fig. 3.6). In addition, the transgene was stable in all the cell types growing in culture, *i.e.*, in cells that had not been transplanted into animals (data not shown). These results strongly suggest that lack of interleukin-8 secretion in tumors that initially had moderate-to-high concentration of interleukin-8 was due to physical “loss” of the transgene and, conversely, the conservation of secretion in tumors that initially had lower concentration of interleukin-8 was due to retention of the transgene.

FIGURE 3.5. Correlation between expression of interleukin-8 and neomycin resistance in cells recovered from MIL-4/MC17-51 mixed tumors.

Spearman rank correlation: $n = 22$, $P < 0.0001$, $r = 0.93$. Other details provided in Materials and methods.

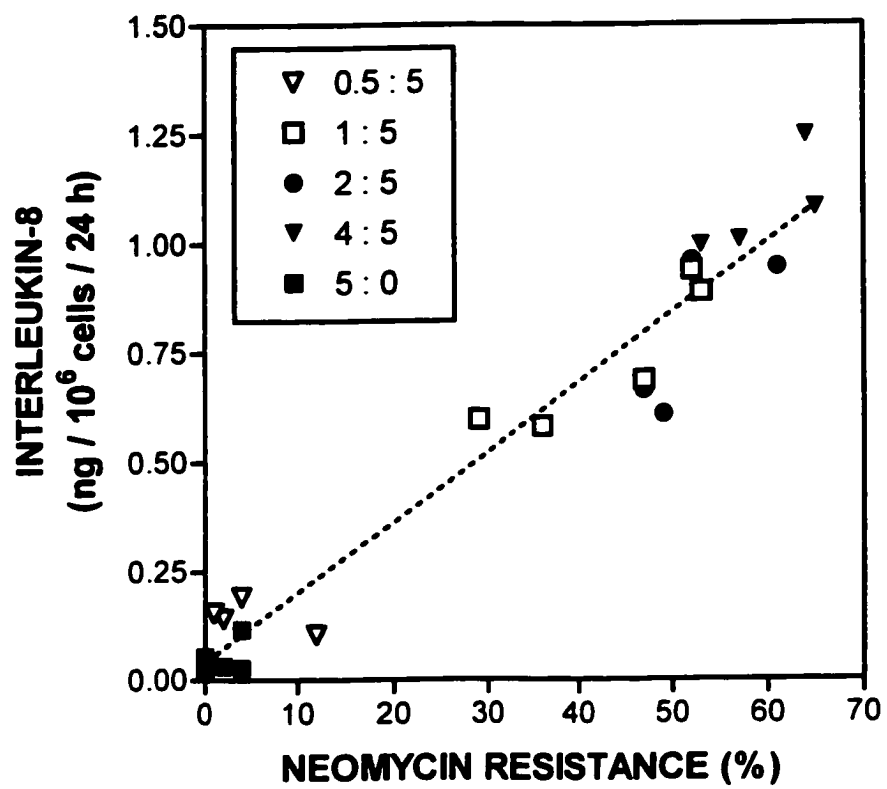
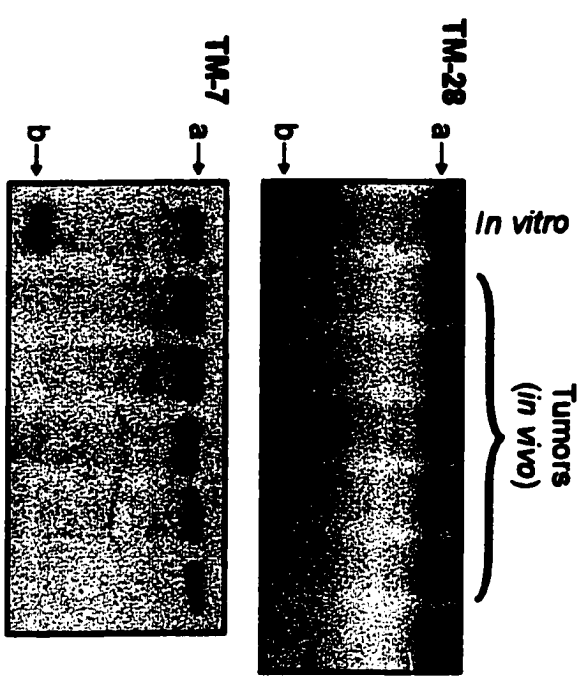
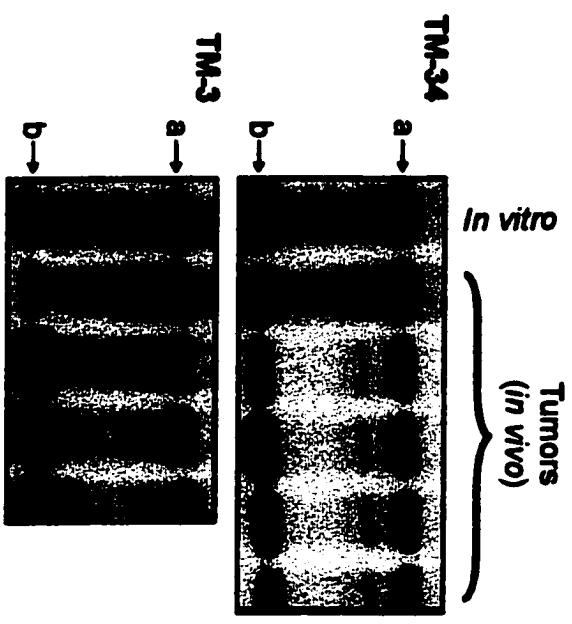
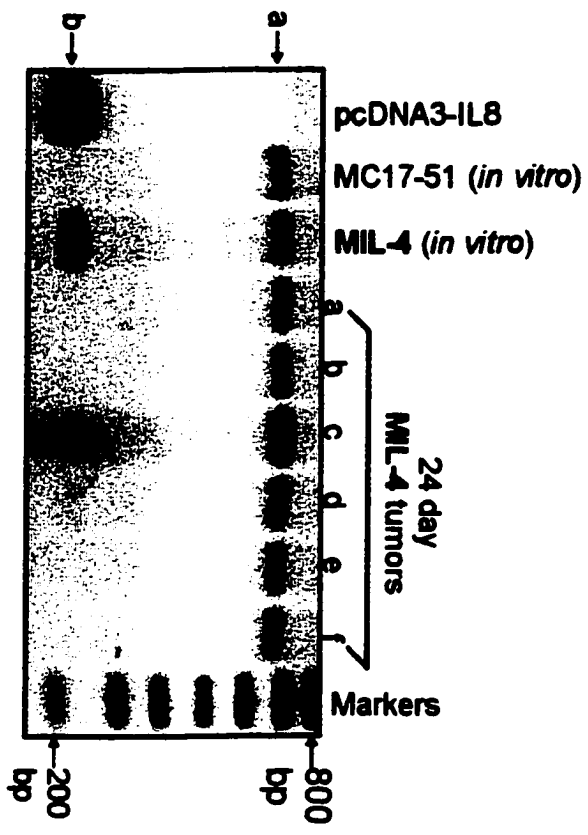


FIGURE 3.6. Status of interleukin-8 transgene in Mutatect MIL-4, TM-34, TM-3, TM-28 and TM-7 tumors as analyzed by PCR analysis.

Genomic DNA was extracted from 3-week old tumors and from same cell-types growing in culture for the equivalent period of time (*in vitro*). The lower band (arrow *b*) corresponds to an interleukin-8-specific fragment, whereas the upper band (arrow *a*) corresponds to non-specific fragment used as an internal control for the PCR reaction. Results are representative of at least 3 independent experiments. Other details as in Materials and methods.



3.7. Discussion

Interleukin-8 injected directly into Mutatect tumors was previously shown to increase neutrophil infiltration. However, this was only seen in 8 out of 36 tumors, likely due to inconsistencies with the injections. Interleukin-8-secreting Mutatect cells were therefore developed in order to allow a persistent source of interleukin-8 in the Mutatect tumors (see *chapter 1*). Five cell lines were developed: one constitutively expressing MIL-4 and four tetracycline-regulatable TM-3, TM-7, TM-28 and TM-34. Large tumors could be produced by TM-28, TM-3, TM-34 and mixtures of MIL-4 and non-interleukin-8 producing MC17-51 cells, whereas very small tumors were produced by TM-7 and MIL-4 cells. In this chapter, we first examined all the tumors for neutrophil content to see if a “continuous” source of interleukin-8 in tumors was capable of consistently recruit a large number of neutrophils. The neutrophil content was measured in the tumors using myeloperoxidase-specific assay described in *chapter 2*. With the exception of MIL-4 tumors, all the interleukin-8-secreting tumors had a higher level of myeloperoxidase than non-secreting tumors. The high level of myeloperoxidase was confirmed to be a result of high neutrophil infiltration. The level of neutrophils could be suppressed by administration of tetracycline in the drinking water of the mice in low interleukin-8-secreting tumors (TM-3 and TM-34) but not in high ones (TM-7, TM-28). In addition, the majority of the tumors formed by each cell type showed consistently high level of neutrophils. This demonstrated that interleukin-8 secretion was able to consistently increase the neutrophil levels in Mutatect tumors.

The neutrophil content in Mutatect tumors did not correlate with the initial interleukin-8 level secreted by the different cell lines. TM-28, a moderate interleukin-8 producer, had the highest neutrophil content, whereas both high and low producers (MIL-4, TM-7, TM-3 and TM-34) had relatively low levels of neutrophils in their tumors. The level of interleukin-8 secreted by TM-28 may be sufficient for an “optimal” recruitment of neutrophils. At higher levels, as in MIL-4 and TM-7 tumors, either interleukin-8 concentration gradient would be disrupted or extensive neutrophil-mediated cytotoxicity/genotoxicity would inhibit tumor establishment (*chapter 1*) and lead to interleukin-8 gene instability (see below). Thus, high neutrophils may not be recruited. This is consistent with the fact that, in MIL-4/MC17-51 mixed tumors, the neutrophil level was significantly enhanced compared to pure MIL-4 tumors. At lower levels of interleukin-8, as

in TM-3, TM-34 and MIL-4/MC17-51 mixed tumors, recruitment of neutrophil may be lower. Therefore, the moderate level of interleukin-8 produced by TM-28 cells may be most favorable for consistently recruiting a high number of neutrophils in Mutatect tumors.

Our laboratory first demonstrated that the *hprt* mutation frequency is 4-fold higher in non-interleukin-8 producing Mutatect cells growing as tumors (*in vivo*) than growing in culture (*in vitro*) and that the level of mutation frequency correlates strongly with the number of neutrophils. In interleukin-8-secreting cells, the *hprt* mutation frequency was significantly higher *in vivo* than *in vitro*. An increase of 5, 8, 15, 35 and 125-fold was respectively observed in MIL-4, TM-3, TM-7, TM-34 and TM-28 cells *in vivo* compared to *in vitro*. The mutation frequency was higher in all interleukin-8-secreting than non-secreting cells *in vivo*. This suggested that the mutagenic effects of the factors in the tumors microenvironment are enhanced in interleukin-8-secreting tumors. When all the tumors were individually analyzed, a very strong correlation between neutrophil content and *hprt* mutation frequency was observed. This suggested that neutrophils are likely the source of the mutagenic factors in the tumor microenvironment.

The stability of interleukin-8 expression was also examined. The expression was stable in all the cell lines *in vitro*. In addition, the expression was stable *in vivo* in cell lines initially expressing low levels of interleukin-8. However, in the cell lines initially expressing high or moderate levels of interleukin-8, the expression was surprisingly absent after 3 weeks of tumor development. This absence of expression was found to be due the "loss" or instability of interleukin-8 transgene. We believe that this is a form of genetic instability and is a result of complex interactions between high level of interleukin-8 producing cells and tumor-infiltrating neutrophils. It does not seem to be due to different sites of integration of the transgene in different cell lines since *in vitro* expression of interleukin-8 by all lines was stable. Rather, we postulate that chemoattraction of a large number of neutrophils early in the development of tumors by a high level of interleukin-8 is responsible. Reactive nitrogen and oxygen species, products of mouse neutrophils (70), can be both genotoxic and cytotoxic (108). The frequency of loss of interleukin-8 *in vivo* was extremely high, approaching 100% in high and moderate expressors; this contrasts with the frequency of mutations at the *hprt* locus, where at its highest it may approach 1%. We therefore postulate that selective cytotoxicity towards interleukin-8-producing cells must be occurring. Since interleukin-8

can delay apoptosis in neutrophils (157), interleukin-8-expressing tumor cells may attract neutrophils while at the same time prolonging their life and extending their cytotoxic capability. The combination of reactive nitrogen oxide-mediated genotoxicity (which fosters loss of the transgene) and selective cytotoxicity towards the remaining interleukin-8-producing cells may explain why cells lacking the transgene eventually dominate the tumor. To explain why tetracycline had an effect in only some tumors, we postulate that tetracycline could not reduce interleukin-8 levels sufficiently in high expressing TM-7 and TM-28 tumors to levels below the chemoattractant threshold. The apparent paradox that the highest interleukin-8 producer, TM-7, showed relatively low neutrophil content and a moderate interleukin-8 producer, TM-28, showed much higher content may be explained by the fact that neutrophils are measured after 3 weeks of tumor growth. TM-7 (and MIL-4) may have attracted a large number of neutrophils at an early stage in tumorigenesis, causing early loss of the interleukin-8 transgene. TM-28 may have attracted fewer neutrophils at an early stage, leading to a delay in loss of the transgene. Since the *hprt* mutation frequency correlates well with neutrophil number determined at the time tumors are harvested, this suggests that the process of mutagenesis occurs throughout tumour development.

The model presented in Fig. 3.7 summarizes some of the complex biological effects that we have observed in Mutatect tumors secreting different levels of interleukin-8. As in *chapter 1*, it is proposed that level of interleukin-8 expression can have a strong influence on tumor biology. At high levels, the predominant effect may be early influx of neutrophils responsible for extensive cytotoxicity and loss of the interleukin-8 transgene. At lower levels, less cytotoxicity and transgene loss may occur, but there may still be a sufficient amount of neutrophil-generated reactive nitrogen oxide species to mediate mutagenicity.

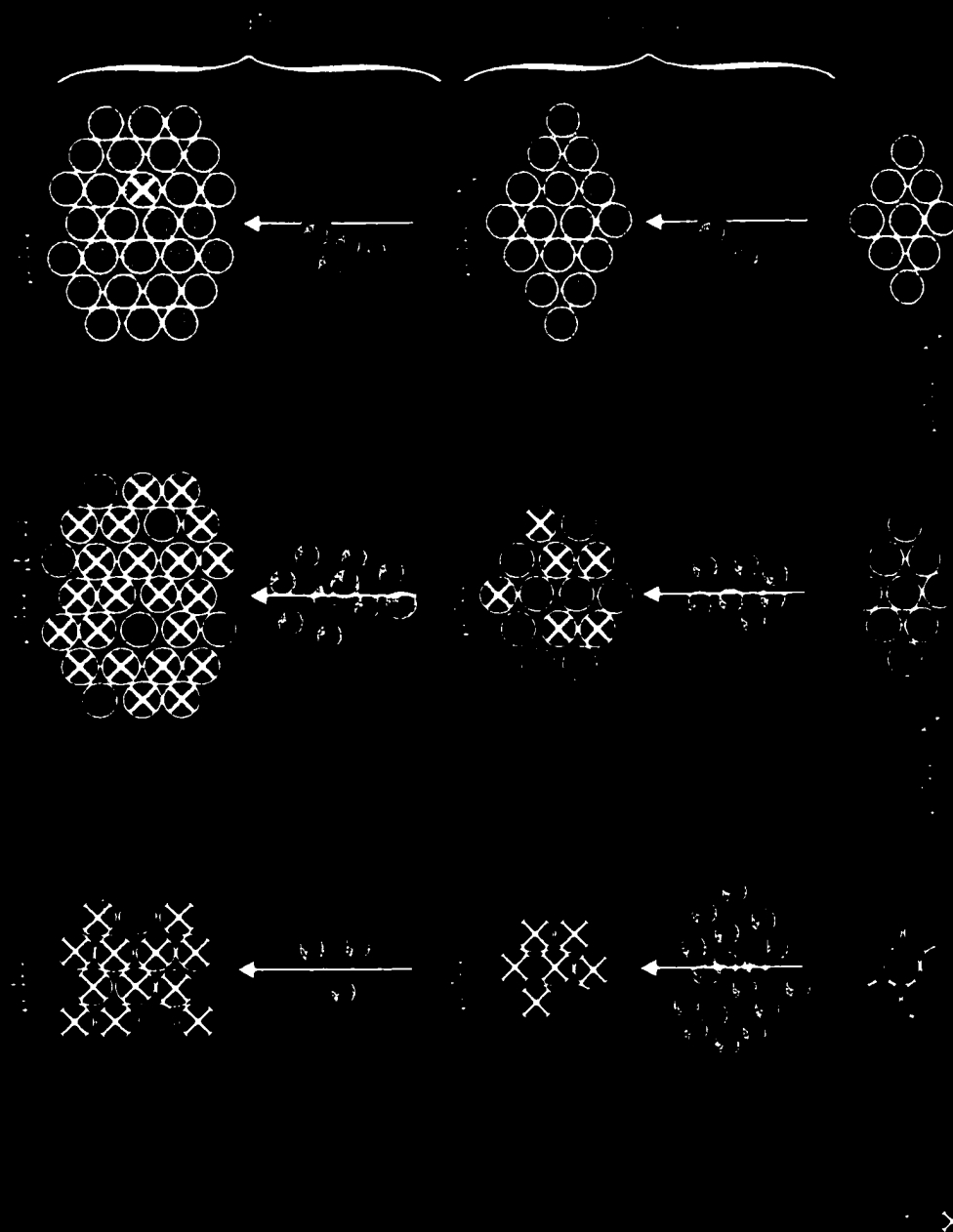
3.8. Significance

Mutatect tumors have been previously shown to be variably infiltrated with neutrophils. Consequently, the *in vivo* *hprt* mutation frequency was also variable. Due to these variabilities, assessment of the effect of neutrophils and neutrophil-derived factors on genetic instability was not very reliable. Mutatect TM-28 cell line, on the other hand, consistently produces tumors with high number of neutrophils and high *hprt* mutation frequency, and also shows a high level of interleukin-8 transgene instability. Thus,

FIGURE 3.7. Model of genetic changes occurring in Mutatect tumors expressing different levels of interleukin-8.

According to this model, high interleukin-8-expressing lines such as TM-7 and MIL-4 attract a large number of neutrophils early in tumor development. This produces high cytotoxicity and a lag in tumor growth. Neutrophil-derived reactive nitrogen oxide species produce genotoxicity, leading to instability in interleukin-8 transgene and enrichment of these interleukin-8-lacking cells for reasons described in the Discussion section. *Hprt* mutations are also induced but there is no enrichment of these mutant cells. Later in tumor development, once interleukin-8-lacking cells dominate the tumor, few neutrophils are attracted. Low interleukin-8-secreting lines such as TM-3 and TM-34 (and mixed tumors) attract relatively few neutrophils early in tumor development. Cytotoxicity and genotoxicity are relatively low, with no instability of interleukin-8 transgene and continued low level attraction of neutrophils both early and late in tumor development. Medium interleukin-8-secreting lines such as TM-28 attract an intermediate number of neutrophils. This produces intermediate genotoxicity, partial instability in interleukin-8 transgene and a low number of *hprt* mutants. Although the instability in interleukin-8 transgene may continue to occur, levels of interleukin-8 are sufficient to continue to attract neutrophils. By the end of the experiments when tumors are harvested, neutrophils are still present and a large number of *hprt* mutants cells have accumulated

Tumor Development



TM-28 tumors are ideal for understanding the effect of high neutrophil infiltration and neutrophil-derived mutagenic factors on *hprt* mutation frequency, transgene instability and other parameters (see *chapters 4 and 5*).

The identification of interleukin-8 transgene instability was also significant because it allowed understanding of the complex biology of tumor development and the role of inflammatory cell in this development. Our proposed mechanism of interleukin-8 transgene instability parallels the accepted mechanism of tumor progression (see *General Introduction*). Both involve genetic instability followed by selection of a clonal population. The possible contribution of neutrophils in transgene instability is consistent with our overall hypothesis that tumor infiltrating inflammatory cells are in some measure responsible for the accumulation of mutation associated with tumor progression.

Most human tumors exhibit genetic instability, the driving force in tumor progression (see *General Introduction*). In addition, many types of human tumors are infiltrated with inflammatory cells such as neutrophils and macrophages. The level of infiltration has been correlated with several parameters associated with tumor progression. Interleukin-8 is also frequently found in many human tumors, and its presence is associated with extensive neutrophil infiltration and poor prognosis (46, 113, 114). Our results show that interleukin-8-producing tumors can have high levels of neutrophil infiltration and consequently a high level of genetic instability. This is a very significant and a novel finding, since it suggests that in the progression of tumors the genetic instability could be, to some extent, a consequence of infiltrating inflammatory cells. We and others have hypothesized that the inflammatory cells contribute to this genetic instability *via* generating reactive oxidants into the tumor microenvironment. This is examined in the following chapters (*chapters 4, 5 and 6*).

3.9. Conclusions

Neutrophils could be consistently chemoattracted in interleukin-8-expressing Mutatect tumors. The number of neutrophils strongly correlated with the number of *hprt* mutations. This strengthens our hypothesis that neutrophils are a source of mutagenic factors. Mutatect TM-28, a moderate interleukin-8 producer, was identified as the cell line capable of producing large tumors with a high number of neutrophils and, consequently, a

high number of *hprt* mutations. In TM-3 and TM-34 tumors, neutrophil levels (and *hprt* mutation frequency) could be manipulated by administration of tetracycline in the drinking water of mice. Finally, interleukin-8 transgene instability was observed in high and moderate interleukin-8 producers *in vivo*, which was likely a consequence of genotoxicity and selective cytotoxicity by neutrophil-derived factors.

**Chapter 4. Effects of dietary vitamin E on genetic instability
and neutrophil infiltration in interleukin-8-secreting Mutatect
tumors**

4.1. Chapter Summary

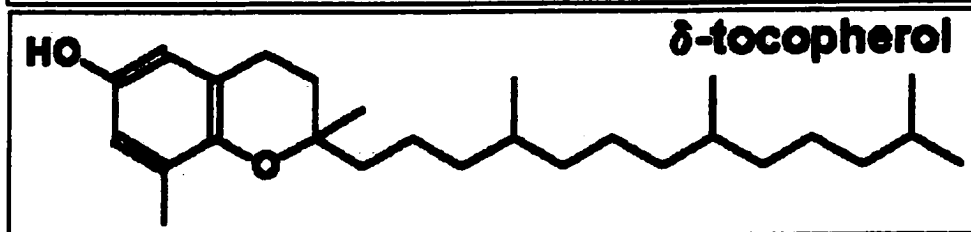
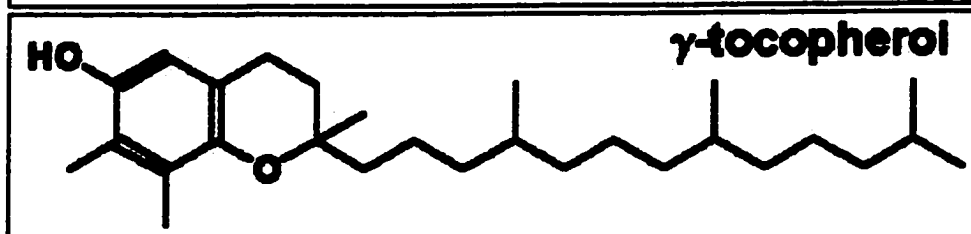
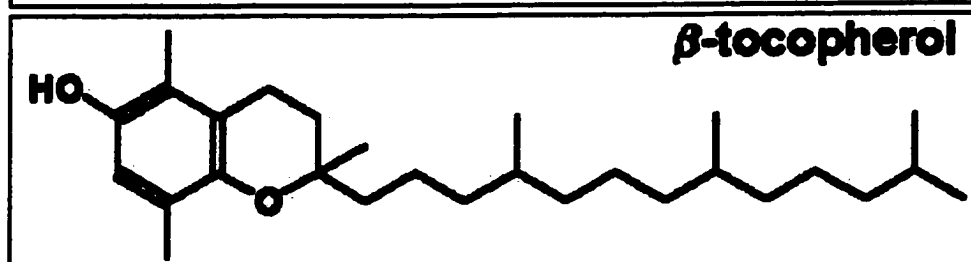
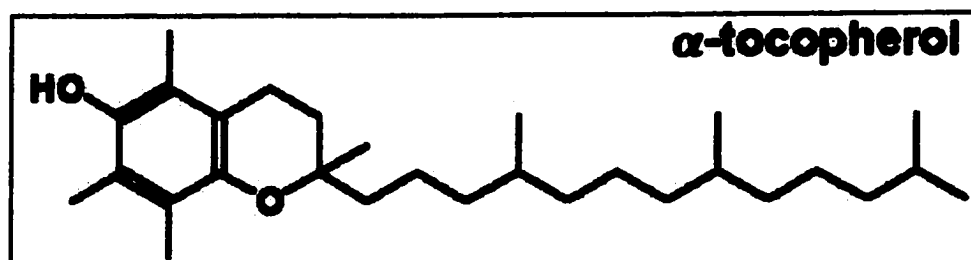
Vitamin E is best known for its ability to scavenge reactive oxygen and nitrogen species. The Mutatect mouse model has been useful in studying the potentially mutagenic role of reactive nitrogen and oxygen species in the tumor microenvironment. Our laboratory has previously shown that Mutatect tumors are infiltrated with neutrophils and that the number of neutrophils correlates with the number of *hprt* mutations and is associated with interleukin-8 transgene instability. Neutrophils are a source of nitric oxide and tumors contain nitrotyrosine, a marker of damage by nitric oxide-related species. The effect of supplementing diets of tumor-bearing mice with vitamin E was also previously tested. A 25% reduction in the *hprt* mutation frequency *in vivo* was found in non-interleukin-8-expressing MN-11 tumors. This suggested a possible involvement of reactive nitrogen and oxygen species in the *in vivo*-arising mutagenicity. In *chapter 3*, we showed that interleukin-8-expressing TM-28 tumors exhibit high neutrophil content, *hprt* mutation frequency and interleukin-8 transgene instability. In this chapter, effects of dietary vitamin E on *hprt* mutation frequency, interleukin-8 transgene instability, neutrophil infiltration, iNOS and nitrotyrosine levels were examined in TM-28 tumors. No effect on inducible nitric oxide synthase expression or nitrotyrosine levels was observed. A ~80% reduction was observed in *hprt* mutation frequency by vitamin E in TM-28 tumors. The *in vivo* instability in interleukin-8 transgene seen earlier in TM-28 tumors was largely prevented. It is evident that at least part of the protective effect of vitamin E was also due to a re-localization of neutrophils within tumors, since vitamin E induced a major redistribution of neutrophils from the “loosely bound” cellular fraction to the “stromal” fraction while the total number of neutrophils in tumors was essentially unchanged. Thus, vitamin E appears to be protective against genotoxicity by scavenging reactive species, but also its ability to affect the distribution of neutrophils within tumors may be important.

4.2. Introduction

Vitamin E is considered to be the most important lipophilic antioxidant in the prevention of cellular injury associated with reactive oxygen and nitrogen species (ROS/NO_x) (91, 92) (see also *General Introduction*). Different forms of vitamin E are found in vegetable oils, of which D- α -tocopherol is considered to be the major biologically active form (Fig. 4.1). Tocopherols are lipophilic molecules that partition into cell membranes where they act as chain-breaking radical scavengers. Dietary supplements of vitamin E (in the form of D- α -tocopherol ester) are widely self-administered in the belief that they can protect against cancer. However, clinical trials of vitamin E supplements or studies of disease risk associated with serum vitamin E levels have failed to show any consistently strong anticancer effect (94-104). An exception appears to be prostate cancer, where there is mounting evidence that vitamin E and other antioxidant micronutrients may be protective (100, 104-106).

The Mutatect mouse model has been useful in studying the potentially mutagenic role of reactive nitrogen oxide species (NO_x) in the tumor microenvironment. Our laboratory has previously shown that tumor-infiltrating neutrophils are a source of NO_x. These neutrophils were shown to express inducible nitric oxide synthase (iNOS). The number of neutrophils and the level of NOS activity in tumors were found to correlate with the *hprt* mutation frequency, a surrogate marker of mutation occurring elsewhere in the genome. In addition, the tumor cells were shown to stain positively for nitrotyrosine, an immunohistochemical marker of peroxynitrite (ONOO⁻) (70). The effect of supplementing diets of tumor-bearing mice with vitamin E was also previously tested. A 25% reduction in the *hprt* mutation frequency *in vivo* was found (107). This suggested the involvement of oxyradicals in mutagenicity. In *chapter 3*, we examined whether neutrophils in fact are a source of mutagenic species in Mutatect tumors. Interleukin-8-secreting Mutatect cells were constructed in an attempt to chemoattract a high number of neutrophils. Mutatect TM-28 cells were identified as capable of consistently chemoattracting a very high number of neutrophils. Consequently, the *hprt* mutation frequency was also very high in these tumors. In addition, these tumors showed a high level of interleukin-8 transgene instability *in vivo*. This genetic instability was probably a consequence of genotoxicity and selective

FIGURE 4.1. Naturally occurring tocopherols.



cytotoxicity by factors produced by neutrophils. Thus, neutrophils are likely a source of mutagenic factors in Mutatect tumors.

To provide additional evidence that neutrophils are inducing a high level of genetic instability *via* reactive oxygen and nitrogen species in Mutatect TM-28 tumors, we examined the protective effect of vitamin E on these tumors in this chapter. The effect of dietary vitamin E on *hprt* mutation frequency, interleukin-8 transgene instability, neutrophil infiltration, iNOS and nitrotyrosine levels was examined in TM-28 tumors.

4.3. Hypotheses

The oxidant-scavenging activity of dietary vitamin E would inhibit *hprt* mutation frequency, interleukin-8 transgene instability and nitrotyrosine levels without affecting iNOS levels and neutrophil infiltration in Mutatect TM-28 tumors.

4.4. Objectives and expected results

The effects of vitamin E on Mutatect TM-28 tumors regarding the following parameters were examined:

1. *Hprt mutation frequency.* Since vitamin E is able to inhibit mutation frequency in Mutatect MN-11 tumors by 25%, a similar reduction in TM-28 tumors was also expected.
2. *Interleukin-8 transgene instability.* In *chapter 3*, it was postulated that neutrophil-derived mutagenic and cytotoxic factors were likely responsible for the instability of the interleukin-8 transgene. Thus, vitamin E was expected to also inhibit this instability.
3. *Neutrophil infiltration.* No effect of vitamin E on neutrophil infiltration was expected. *iNOS and nitrotyrosine levels.* No effect of vitamin E on iNOS level was expected.

Since nitrotyrosine is a marker of NO_x-dependent damage, vitamin E was expected to reduce nitrotyrosine levels in tumors.

4.5. Materials and Methods

Mutatect cell culture, tumor formation and tumor fractionation. Conditions for cell culture, detection of *hprt* mutants and removal of pre-existing mutant cells prior to formation

of subcutaneous tumors have also been reported in *section 3.5*. Tumors were formed by subcutaneous injection of Mutatect TM-28 cells into C57BL/6 mice and excised after about 3 weeks, as described in *section 1.5*. About 25 % of each tumor was used for histological analysis, isolation of DNA for PCR analysis of interleukin-8 transgene and vitamin E analysis. The remainder of the tumor was gently minced in 5 ml of Dulbecco's modified Eagle's medium plus 10% fetal calf serum and mechanically dispersed by passage through a 3 cc-syringe. The suspension was incubated in a 15 ml tube at 0°C for 3-5 min to allow the particulate (stromal fraction) to settle. The supernatant (cellular fraction) was separated from the particulate. The terms "cellular" and "stromal" fractions are operational definitions. Microscopically, the cellular fractions consist of single cells or small clumps of cells, whereas stromal fraction consists of fragment of tissues, including connective tissue and blood vessels. Both fractions were analyzed for myeloperoxidase activity, number of *hprt* mutations, protein content and DNA content. Fluorometric methods employing fluorescamine (147, 148) and diaminobenzoic acid (158) were used for quantification of protein and DNA, respectively. All experiments using animals were carried out at the Animal Care & Veterinary Service of the University of Ottawa in accordance with guidelines of the Canadian Council on Animal Care.

Dietary supplements with vitamin E. Mice were fed a control, vitamin E-rich or a vitamin E-stripped diet. Animals on control rodent diet (Charles River) received 0.45 IU of D- α -tocopherol per day. To make vitamin E-rich chow (2.45 IU/day), 2 IU of D- α -tocopherol acetate (Ciba-Geigy; diluted in soy oil) was added to each 5 gram pellet of chow. Vitamin E-stripped chow was obtained from ICN Biochemicals Inc. (Aurora, OH). Vitamin E supplements were started 7 days prior to tumor cell injection and continued until animals were sacrificed. No effect was observed of vitamin E supplements on mice behaviour or survival, or on tumor volume. Other details are described elsewhere (107).

Analyses of myeloperoxidase activity, hprt mutations and interleukin-8 transgene instability. Details of these analyses are described in *section 3.5*. Myeloperoxidase activity and *hprt* mutations were measured in both cellular and stromal fractions of tumors. In experiments where the *hprt* mutation in the stromal fraction of tumors was estimated, small fragments of tumor were incubated in medium for 2 d, and then removed. The *hprt* mutation

frequency of those cells that remained attached to the dishes was determined as described for cellular fraction in *section 3.5*.

Western Blot Analysis for nitrotyrosine. See *chapter 6* for complete analysis.

Nitrotyrosine Immunohistochemistry. This was carried out by Jagdeep K. Sandhu. Formalin-fixed tumor tissue sections were deparaffinized and heated at 90–100°C for 12 minutes in 0.01 mol/L sodium citrate, pH 6.0. Endogenous peroxidase activity was inactivated using 0.5% hydrogen peroxide in methanol for 30 min at room temperature. Nonspecific immunoglobulins were blocked with 1% normal goat serum at room temperature for 30 minutes. Excess liquid was drained and sections were incubated in a humid chamber with 2.5 µg/ml of a rabbit polyclonal antibody to nitrated KLH (Upstate Biotechnology, Lake Placid, NY). Sections were incubated for 1 hour at room temperature followed by incubation for 30 minutes with DAKO Envision peroxidase conjugated to goat anti-rabbit/anti-mouse Ig antibody (DAKO Corp., Carpinteria, CA). Immunolabeling was detected using DAB as the chromogen, washed and counterstained with Mayer's hematoxylin. Tests of the specificity of the antibody for nitrotyrosine were (i) preincubation with either 10 mmol/L nitrotyrosine (Sigma) or (ii) 50 µg/ml nitrated bovine serum albumin (prepared by treatment of albumin with sodium nitrite and H₂O₂) or (iii) reduction of tissue nitrotyrosine to aminotyrosine with sodium hydrosulfite (70).

Statistical Analysis. Non-parametric statistical tests were used. The Mann-Whitney U test was used to compare 2 unpaired groups. The Kruskal-Wallis test was used to compare 3 or more unpaired group. Spearman rank correlation was used to determine a correlation between 2 measured variables. A value of $P < 0.05$ was considered to be statistically significant. In the figures, P -values between 0.01 and 0.05 is represented by * and P -values between 0.001 and 0.01 is represented by **.

4.6. Results

4.6.1. *Hprt* mutation frequency in TM-28 tumors after vitamin E administration

Previous results from our laboratory have shown that dietary vitamin E is able to reduce the *hprt* mutation frequency in non-interleukin-8-producing Mutatect MN-11 tumors by 25% (107). We recently developed interleukin-8-secreting Mutatect TM-28 cells that produce tumors with high number of both infiltrating-neutrophils and *hprt* mutation

frequency (*chapter 3*). To examine whether vitamin E would also be as protective in these tumors, we formed subcutaneous TM-28 tumors in mice that were maintained on either a control (0.45 IU vitamin E/day) or a vitamin E-rich (2.45 IU vitamin E/day) diet and analyzed them for *hprt* mutation frequency. The cellular fraction of the tumors was used for this analysis. The tumors and plasma from mice were first analyzed for vitamin E levels to determine whether the mice are receiving vitamin E. Both the plasma and tumoral vitamin E were significantly higher in the mice fed the vitamin E-rich diet than controls (Table 4.1). A marked effect of vitamin E supplementation on *hprt* mutation frequency was observed (Fig. 4.2). In two separate experiments, the median mutation frequency of the vitamin E group was statistically significantly lower than that of the control group: 84% lower in experiment 1 ($P = 0.006$) and 81% lower in experiment 2 ($P = 0.005$)⁵. Thus, vitamin E was protective against *in vivo*-arising mutations in TM-28 tumors, which have high neutrophil content, and the extent of this protection was significantly more than the original non-interleukin-8-secreting MN-11 tumors, which have low neutrophil content.

4.6.2. Interleukin-8 transgene instability in TM-28 tumors after vitamin E administration

We have earlier shown that Mutatect cell lines that initially express moderate-to-high level of interleukin-8 (e.g., TM-28) show instability in the interleukin-8 transgene when the cells were growing as tumors but not in culture. It was postulated that a combination of genotoxicity and selective killing by neutrophil-derived oxidants was responsible for the instability of the transgene (*Chapter 3*). Since dietary vitamin E was protective against the *hprt* mutations in Mutatect tumors, its effect on transgene stability was tested using Mutatect TM-28 tumors. Genomic DNA was isolated from tumors and analyzed by PCR for an interleukin-8-specific fragment. In agreement with the earlier reported results (see section 3.6.4), the interleukin-8-specific fragment could not be detected in any of the 6 tumor DNAs from the control group (Fig. 4.3, top). In striking contrast, DNA from all 6 tumors from vitamin E-treated group retained the interleukin-8 specific sequences (Fig. 4.3, bottom). Very similar results were obtained in a second experiment; the interleukin-8-specific fragment was present in 1 of 8 tumors of the control group and in 6 of 7 tumors of the

⁵ Similar inhibition has also been observed in at least 5 other experiments.

TABLE 4.1: α -Tocopherol levels in plasma and tumors of mice receiving a control diet (0.45 IU α -tocopherol/day) or a supplemented diet (2 IU α -tocopherol/day)^(a)

Diet	α -Tocopherol levels			
	Plasma (nmol/ml)		Tumors (nmol/g wet wt.)	
	Median	Confidence Interval	Median	Confidence Interval
Control	5.54	4.50-6.57	15.23	8.77-19.15
Supplemented	8.86	6.88-9.61	21.60	14.12-30.74

^(a) Levels of α -tocopherol were measured using HPLC analysis by Dr. N. Hidioglou and C. Soo.

FIGURE 4.2. Effect of dietary vitamin E on *hprt* mutation frequency in Mutatect TM-28 tumors.

Mice were either maintained on a control diet (0.45 IU vitamin E per day) or a vitamin E-supplemented diet (2.45 IU per day). Other details provided in Materials and methods. Each point represents a tumor

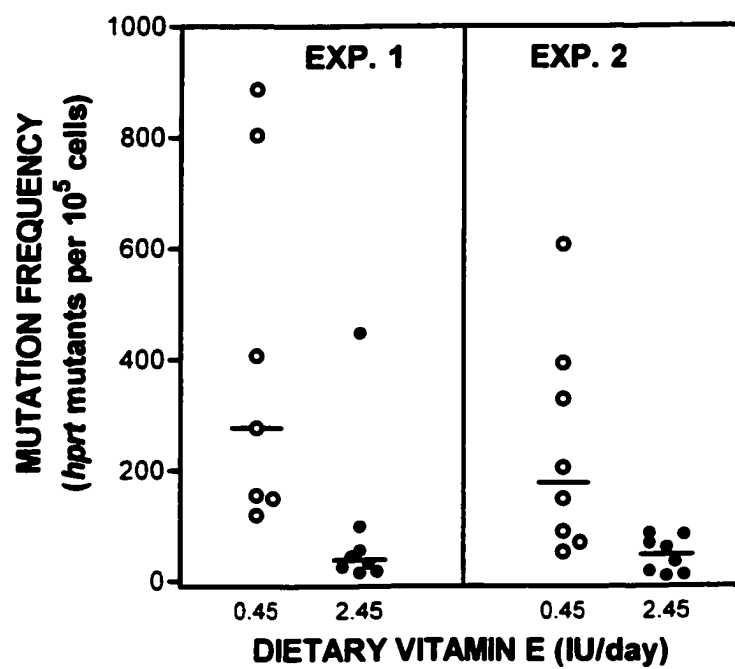
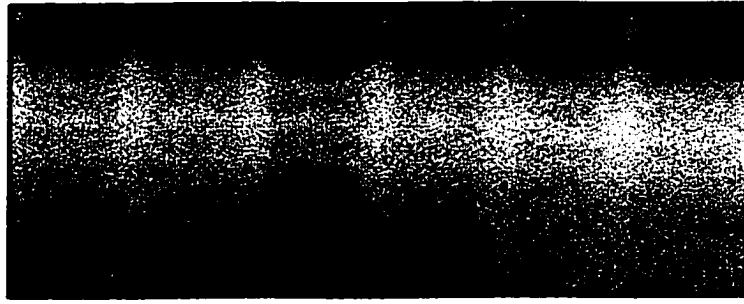


FIGURE 4.3. Effect of dietary vitamin E on *in vivo* transgene instability of interleukin-8 in Mutatect TM-28 tumors.

The transgene was detected by PCR analysis in tumor DNA from animals receiving a control diet (0.45 IU vitamin E/day) or a vitamin E-rich diet (2.45 IU/day). The lower band (arrow b) corresponds to an interleukin-8-specific 241 bp fragment, while the upper band (arrow a) corresponds to an uncharacterized 670-bp fragment used as an internal control for PCR efficiency.

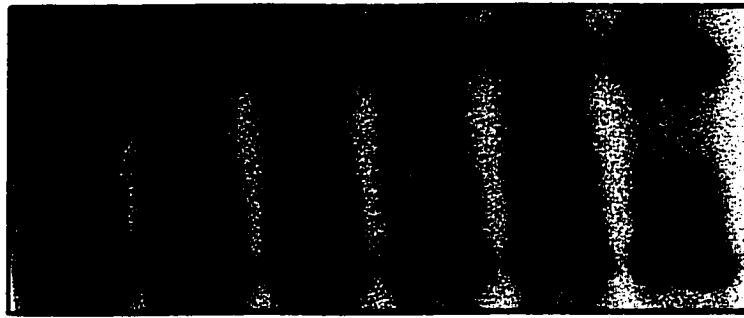
Control



← **a**

← **b**

Vit E



← **a**

← **b**

vitamin E-treated group (data not shown). Thus, dietary supplements with vitamin E profoundly inhibited the instability of the interleukin-8 transgene in TM-28 tumors.

4.6.3. Neutrophil status in TM-28 tumors after vitamin E administration

Mutatect TM-28 tumors are characterized by high number of infiltrating-neutrophils (*Chapters 3*). To examine whether vitamin E would affect neutrophil content in these tumors, we measured the levels of myeloperoxidase (a neutrophil-specific marker) to estimate neutrophil infiltration in TM-28 tumors from mice that had been fed either a control or a vitamin E-rich diet. The cellular fraction of the tumors was initially used for the analysis, that is, the fraction released by gentle homogenisation of tumors with a syringe. Surprisingly, a marked effect of vitamin E on myeloperoxidase levels was observed. Myeloperoxidase levels were 76% lower in tumors from vitamin E-supplemented animals than in the control group in experiment 1 ($P = 0.04$) and 72% lower in experiment 2 ($P = 0.0006$) (Fig. 4.4A). To determine whether the apparent decrease in the myeloperoxidase content in the vitamin E-group was due to a decrease in neutrophil influx, tumors were counted microscopically in histological sections. Contrary to the myeloperoxidase results, only a small, not statistically significant, difference in the microscopically-counted neutrophil number between the vitamin E-supplemented group and the control group was observed (Fig. 4.4B). Since myeloperoxidase levels were measured in the 'cellular fraction', additional experiments were carried out to also determine myeloperoxidase levels in the 'stromal fraction', that is, the tumor fragments remaining after homogenisation. The distribution of myeloperoxidase both in the cellular and stromal fractions was measured. As found above, the myeloperoxidase level in the cellular fraction was significantly reduced ($>80\%$, $P = 0.002$) in the vitamin E-group compared to the control group (Fig. 4.5). By contrast, the myeloperoxidase level in the stromal fraction was *not* decreased (Fig. 4.5); rather, it was significantly increased (2-fold, $P = 0.008$). In the vitamin E-group, the cellular myeloperoxidase level was 7-fold lower than in the stroma ($P = 0.003$), while in the control group, little or no difference in myeloperoxidase in the two fractions was observed. This suggested that increased dietary vitamin E led to a redistribution of neutrophils within tumors rather than a decrease in number of tumor-infiltrating neutrophils. As an estimate of cell

FIGURE 4.4. Effect of dietary vitamin E on neutrophil infiltration into Mutatect tumors.

A) Myeloperoxidase activity was measured in the cellular fractions of tumors from animals receiving a control diet (0.45 IU/day) or a vitamin E-rich diet (2.45 IU/day). **B)** Tumor-infiltrating neutrophils microscopically counted in histological sections of paraffin-embedded tumors. Counting was carried out by Jagdeep K. Sandhu as described in chapter 2. Results were expressed as the mean number of neutrophils per field. Each point represents a tumor, and the bar represents the median value. Other details as in Materials and Methods.

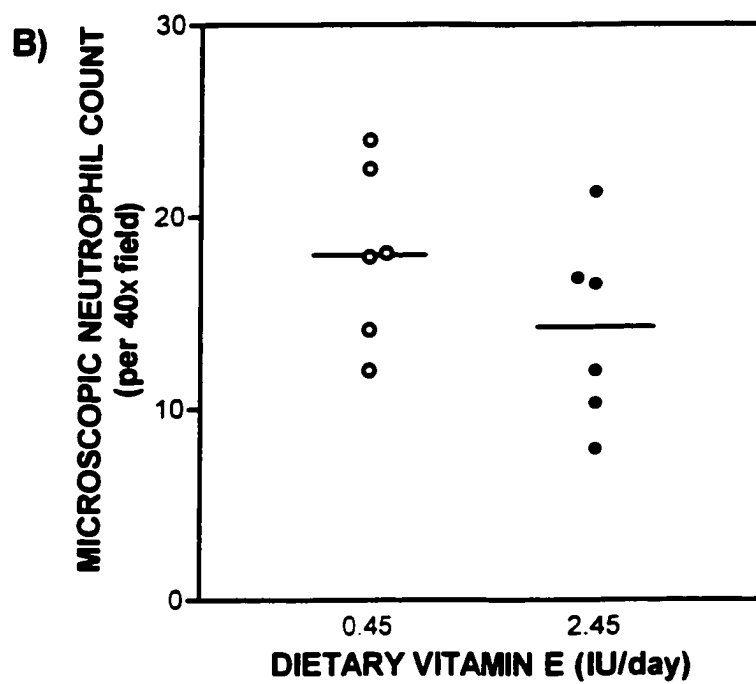
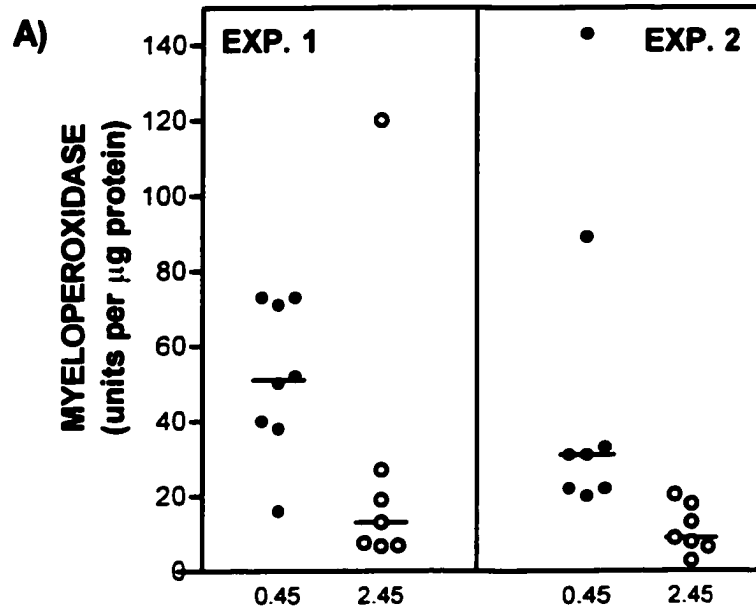
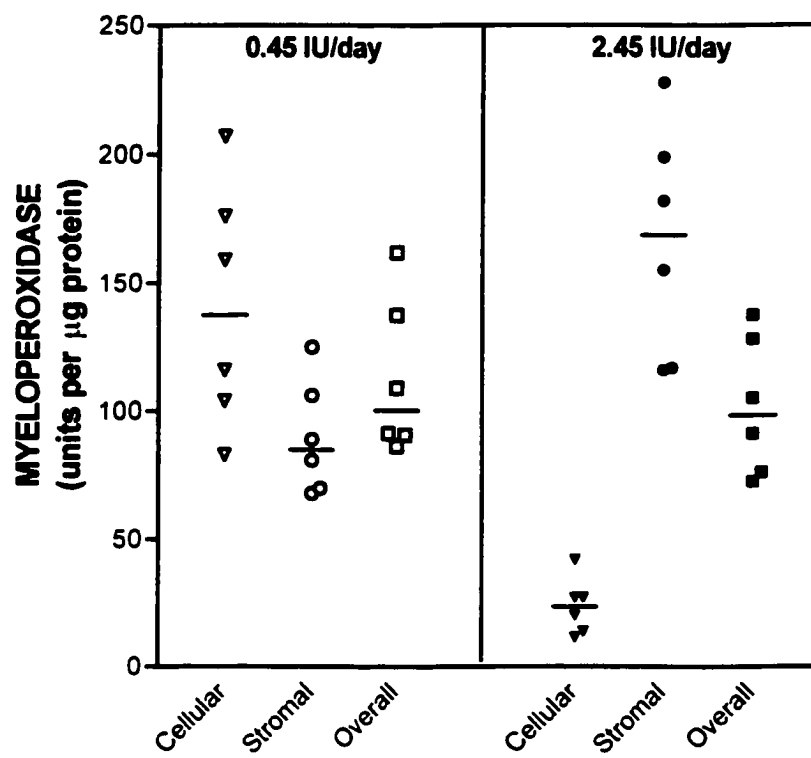


FIGURE 4.5. Neutrophil redistribution as a result of dietary vitamin E in Mutatect TM-28 tumors.

Myeloperoxidase activity was measured in the both the cellular and the stromal fractions of tumors from animals receiving a control diet (0.45 IU/day) or a vitamin E-rich diet (2.45 IU/day). Overall represents the level of myeloperoxidase in the total tumor (calculated from the sum of myeloperoxidase in the cellular and stromal fractions). Each point represents a tumor, and the bar represents the median value. Other details as in Materials and Methods.



number in the two fractions, cellular DNA was measured. No significant difference between the two groups in the percentage of cells in the cellular fraction was observed: $45 \pm 11\%$ and $48 \pm 7\%$ (mean $\pm$ SEM) in the control (n=6) and vitamin E (n=6) groups, respectively. Thus, the lower level of myeloperoxidase in the cellular fraction of the vitamin E-rich group was not due to a reduced extractability of cells. Total tumor myeloperoxidase activity (cellular + stromal) was not significantly different in the two groups (Fig. 4.5). This is in agreement with the histological estimate of the total tumor neutrophils (Fig. 4.4B). This conclusion is further substantiated by a second experiment in which a vitamin E-stripped (0 IU), control (0.45 IU) or vitamin E-supplemented (2.45 IU) diet was used (Fig. 4.6A). We conclude that dietary vitamin E had a marked effect on the localization of neutrophils within Mutatect tumors *without* affecting the total neutrophil influx.

4.6.4. Stromal *hprt* mutation frequency in TM-28 tumors after vitamin E administration

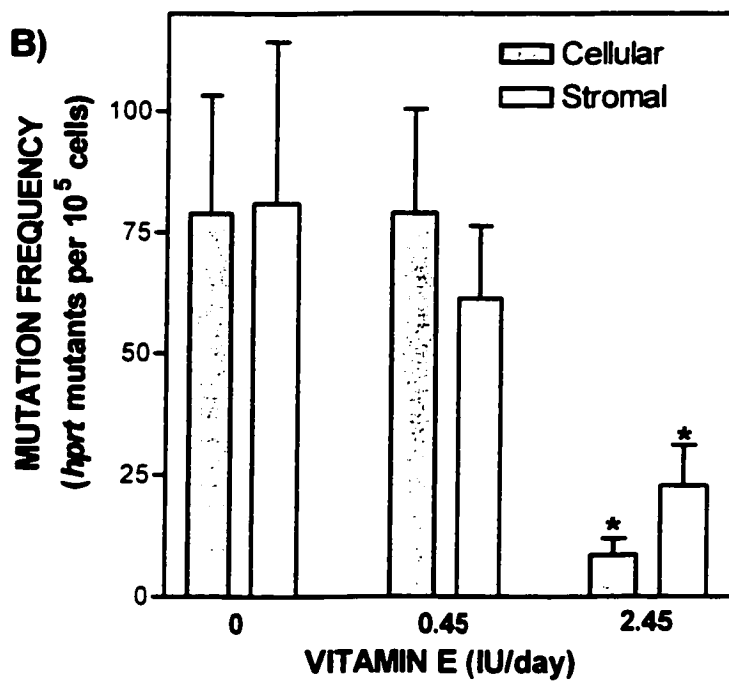
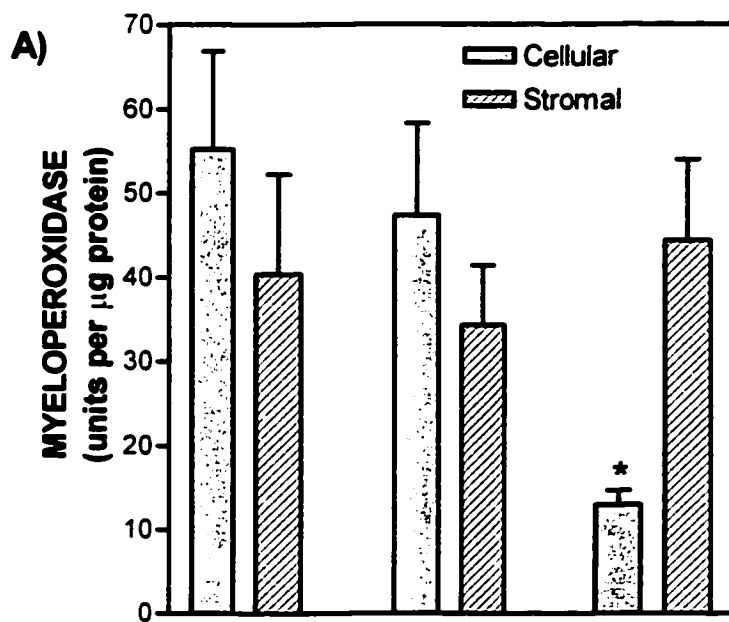
Dietary vitamin E was able to dramatically reduce the *hprt* mutation frequency in TM-28 tumors (see Fig. 4.2). Since the mutation frequency in those experiments was measured in the cellular fractions of Mutatect tumors only, we examined whether the distribution of the mutation frequency in both tumor fractions was also affected (Fig. 4.6B). In agreement with the above results, the *hprt* mutation frequency in the cellular fraction was significantly lower in the vitamin E-supplemented group (2.45 IU) than in the control group (0.45 IU) or the vitamin E-stripped group (0 IU) ($P < 0.05$ for both, Kruskal-Wallis test). There was also a statistically significant decrease in the mutation frequency in the stromal fraction of the vitamin E-supplemented group compared to the other groups ($P < 0.05$ for all; Fig. 4.6B). In the cellular fraction of the high dose vitamin E group, the decrease in mutation frequency was associated with the decrease in the myeloperoxidase level. By contrast, in the stromal fraction the decrease in mutation frequency occurred despite a lack of change in the myeloperoxidase level (Fig. 4.6A). Thus, dietary vitamin E supplements decreased the mutation frequency in all fractions of the tumor.

4.6.5. iNOS and nitrotyrosine levels in TM-28 tumors after vitamin E administration

Tumor-infiltrating neutrophils express iNOS, a source of nitric oxide (70). To address the question of whether the effect of vitamin E on tumors was due to a down-

FIGURE 4.6. Dose-response curve for dietary vitamin E on neutrophil (myeloperoxidase) level and *hprt* mutant frequency in Mutatetect TM-28 tumors.

A) Myeloperoxidase activity was measured in the cellular and stromal fractions of tumors from animals receiving a vitamin E-stripped diet (0 IU/day), a control diet (0.45 IU/day) or a vitamin E-rich diet (2.45 IU/day). **B)** *Hprt* mutation frequency was measured in the cellular and stromal fractions of tumors from the same groups of animals. The bars represent the mean $\pm$ SEM of 6 separate tumors per group. Other details as in Materials and Methods.

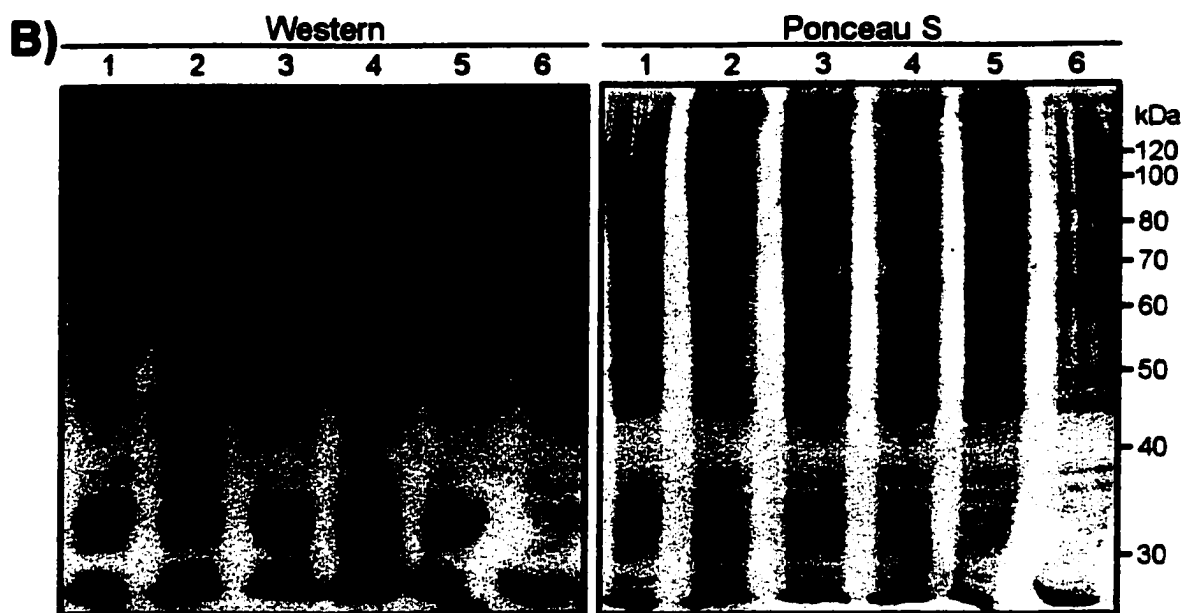
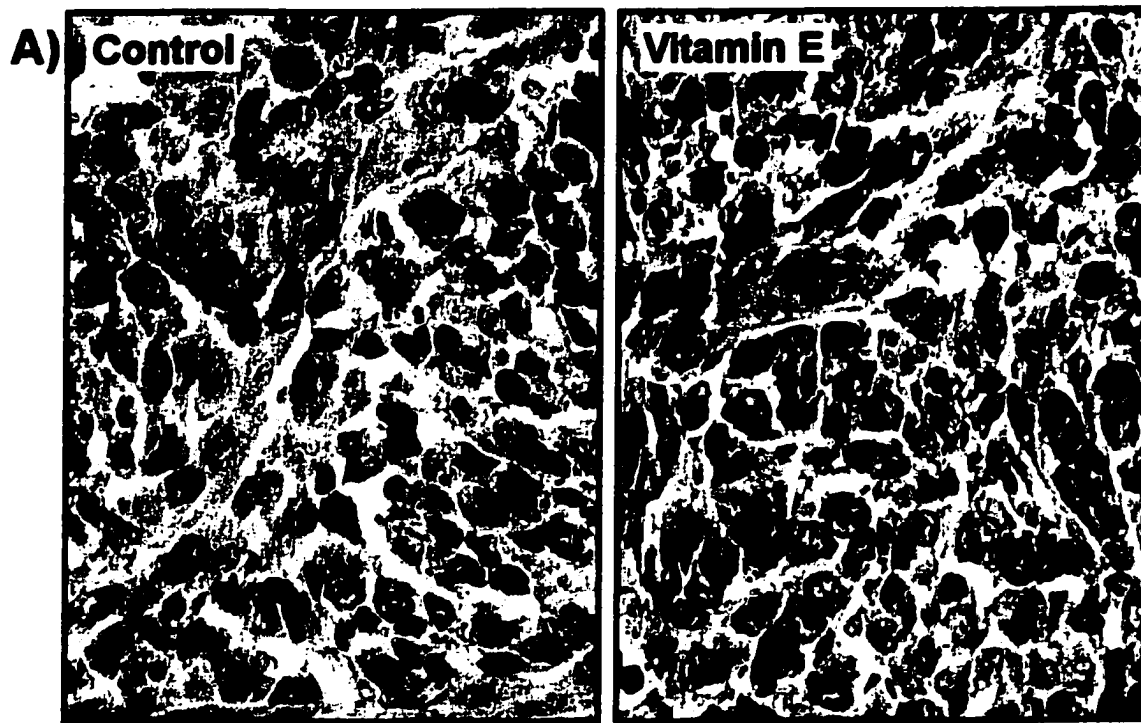


regulation of iNOS, immunohistochemical staining for iNOS was carried out on tumors from the control and vitamin E-supplemented groups. As reported previously, staining was mainly seen in neutrophils, although some macrophages at the periphery of the tumor also stained. Notably, no difference in staining pattern or intensity between the control and vitamin E groups was observed (data not shown).

Nitrotyrosine, a marker of peroxynitrite-based cell-damage, was also seen in Mutatect tumors (70)(*chapter 5*). To determine the effect of dietary vitamin E supplements on this form of protein modification, immunohistochemical staining and western blot analysis for nitrotyrosine was carried out. Widespread staining of tumors, including tumor cells, neutrophils and macrophages, was observed (Fig. 4.7A). No discernible difference was seen in the pattern or intensity of staining between the two groups. Similarly, no difference was seen between control and vitamin E groups by western blot analysis of tumor extracts, although nitration of many proteins was seen in both (Fig. 4.7B). The specificity of staining by the anti-nitrotyrosine antibody on western blots is demonstrated in *Chapter 5*. Thus, these results suggest that dietary vitamin E had no effect on iNOS expression and protein nitrotyrosine formation.

FIGURE 4.7. Detection of nitrotyrosine in Mutatect tumors from mice receiving a control diet (0.45 IU vitamin E/day) or a vitamin E-rich diet (2.45 IU/day).

A) Immunohistochemical detection of nitrotyrosine in paraffin-embedded tumor sections. Carried out by Jagdeep K. Sandhu. The sections were counterstained with hematoxylin. Magnification $\times 400$. **B)** Western blot detection of nitrotyrosine in tumor homogenates (20 mg/per lane) from 3 representative control (*lanes 1-3*) and 3 vitamin E (*lanes 4-6*) group tumors. The same blots were Ponceau S stained prior to western analysis for comparison. No obvious difference in protein nitrotyrosine between control and vitamin E groups by either immunohistochemistry or Western blotting was observed. Other details as in Materials and Methods.



4.7. Discussion

Mutatect TM-28 cells were constructed to study the effects of potentially mutagenic factors derived from tumor-infiltrating neutrophils on genetic instability *in vivo*. TM-28 cells are capable of producing subcutaneous tumors with a high number of neutrophils and a high level of *hprt* mutation frequency and interleukin-8 transgene instability. We have shown that neutrophils are likely the source of mutagenic factors using this and other interleukin-8-secreting cell lines (*chapter 3*). Previously, our laboratory has shown that neutrophils are a source of reactive NO_x species, which are potentially cytotoxic and mutagenic. In this chapter, we set out to demonstrate whether the neutrophil-dependent genetic instability in TM-28 tumors is due to oxidants (e.g., NO_x) released by neutrophils. Thus, the effect of dietary antioxidant vitamin E was studied in these tumors. It was found to be dramatically protective.

It is evident that at least part of the protective effect of vitamin E was also due to a re-localization of neutrophils within tumors. The decrease in neutrophil content in the cellular fraction (i.e., single cells released from the tumor by gentle homogenization) was accompanied by an increase in myeloperoxidase in the stromal fraction (which contains blood vessels, connective tissue and about 60% of total cellular DNA). This redistribution of neutrophils from the cellular to the stromal fraction was dose-dependent: there was little difference between animals on a vitamin E-stripped diet and regular chow (0.45 IU/day) but there was a marked effect on animals consuming chow containing 2.45 IU/day. The high dose of dietary vitamin E did not change the total tumor burden of neutrophils, nor did it have a discernible effect on iNOS expression or on protein nitrotyrosine levels. Although vitamin E is best known for its antioxidant/radical-scavenging properties (93), it appears to exert other biological effects that are not as widely appreciated (91). It can affect the extravasation of leukocytes into tissues by selectively reducing expression of cell adhesion molecules, perhaps by inhibiting the activation of nuclear factor κ B (159-168). The adhesion molecules E-selectin, intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), CD11b/CD18 and very late antigen-4 (VLA-4) are known to increase the adhesiveness of neutrophils to endothelium. Thus, it is possible that the redistribution of neutrophils within Mutatect tumors caused by vitamin E supplements is due

to an alteration of the expression of cell adhesion molecules on neutrophils, tumor cells or other types of cells in the tumor.

Previous results from our laboratory have shown that dietary vitamin E is able to reduce the *hprt* mutation frequency in *non*-interleukin-8-producing Mutatect MN-11 tumors by 25% (107). In addition, vitamin E prevented the *hprt* mutations induced by such NO_x-donating compounds as glyceryl trinitrate and molsidomine in MN-11 tumors (107). MN-11 tumors have a low neutrophil content while TM-28 tumors have a very high neutrophil content. We here show a ~80% reduction in *hprt* mutation frequency by vitamin E in TM-28 tumors. The reduction in mutations was seen in both cellular and stromal fractions, while a reduction in neutrophils was only seen in the cellular fraction. Not only does this confirm that the majority of the *hprt* mutations is caused by neutrophils in TM-28 tumors, but it also suggests the involvement of reactive oxidants in the mutagenic process. Possible involvement of neutrophils and/or oxidants was also shown for the instability of interleukin-8 transgene. This complemented our earlier postulate that the transgene instability is a result of genotoxicity and selective killing by neutrophil-derived oxidants. Since reactive NO_x are potentially mutagenic (see *General Introduction*), we believe that neutrophil-derived NO_x are responsible for the high level of genetic instability in TM-28 tumors.

Protein nitrotyrosine levels did not appear to be affected by dietary vitamin E as determined by immunohistochemistry and western blotting. A more quantitative method (e.g. ELISA, mass spectrometry) needs to be used to more reliably address this. Other researchers have shown a large decrease in nitrotyrosine levels by vitamin E administration in other animal disease models (169, 170). The lack of a detectable decrease in our experiments does *not* rule out the involvement of reactive NO_x species in genetic instability (see also discussion of *chapter 6*), since not all mutagenic NO_x species induce nitrotyrosine formation. Nitrotyrosine is a marker of the presence of ONOO⁻ or myeloperoxidase-derived NO₂Cl (see *General Introduction*). A better demonstration of the involvement of these and other NO_x species in genetic instability would be to examine the effect of more specific scavengers of NO_x and/or use of NOS inhibitors. Since part of the protective effect of vitamin E appeared to be the redistribution of neutrophils in the tumors, this effect may be more important than the classical scavenging effect of vitamin E in protecting genetic instability in the Mutatect tumors.

4.8. Significance

The significant protective effects of dietary vitamin E observed in this chapter have allowed us to better understand the mechanism of genetic instability in Mutatect tumors. It further confirms that neutrophils are contributing to the high *hprt* mutation frequency *in vivo* and demonstrates that reactive oxidants (derived from the neutrophils) are likely involved. Another significant finding is that interleukin-8 transgene instability is likely a consequence of neutrophil infiltration and reactive oxidants. The dramatic effect on neutrophil redistribution suggests that the protective effect of vitamin E is complex. Thus, the non-antioxidant effect of vitamin E should also be taken into account when examining the protective effect of vitamin E.

Most human tumors exhibit genetic instability. We have so far shown that genetic instability could be enhanced in tumors by recruiting a high number of neutrophils and inhibited by administering vitamin E. Dietary supplements of vitamin E are widely self-administered in the belief that they can protect against cancer. Although in some instances some protective effects have been observed, most clinical trials of vitamin E supplements or studies of disease risk associated with serum vitamin E levels have failed to show any strong anticancer effect. Based upon our results, we postulate that vitamin E would be more protective in the tumors that have high levels of inflammatory cell infiltration. In collaboration with Dr. Susan Robertson at the Ottawa Hospital General Campus, our laboratory has recently examined the level of inflammatory cell infiltration in the prostate of prostate cancer and control individuals. This type of cancer has been shown to be consistently protective by vitamin E (100). A high level of inflammatory cell infiltration (both neutrophil and macrophages) was seen in the prostate of cancer patients compared to control individuals (unpublished data).

4.9. Conclusions

Dietary vitamin E dramatically protected against high *hprt* mutation frequency and interleukin-8 transgene instability in Mutatect tumors (TM-28) that have high levels of infiltrating neutrophils. In addition, dietary vitamin E had a marked effect on the localization of neutrophils within the tumors. Thus, the protective effect may be due to two separate

mechanisms. Vitamin E may disrupt neutrophil-tumor cell interaction important in genotoxicity and/or it may scavenge NO_x produced by neutrophils.

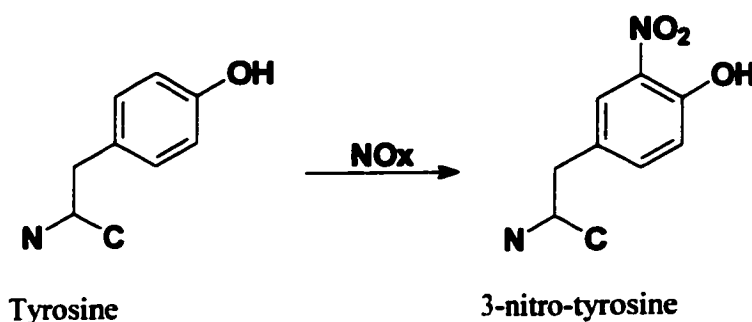
Chapter 5. Detection and identification of nitrotyrosine-containing proteins as a marker of reactive NO_x-mediated damage in interleukin-8-secreting Mutatect tumors

5.1. Chapter Summary

Protein nitrotyrosine is a cellular marker for the presence of nitric oxide-derived reactive nitrogen oxide species. Using immunohistochemistry, we have previously detected nitrotyrosine in murine Mutatect tumors, where neutrophils are the principal source of nitric oxide. We now report on the identification of prominent nitrotyrosine-containing proteins in tumor extracts. Proteins ≤ 15 -kDa consistently stained for nitrotyrosine, as detected by western blot analysis. The overall nitrotyrosine level was highest in tumors with the highest number of neutrophils. Little protein-nitrotyrosine was seen in Mutatect cells growing *in vitro*. However, extended (>72 h) but not shorter (24 h) treatments of cultured cells with nitric oxide-donating drugs caused nitration of ≤ 15 -kDa proteins. These proteins, from both tumors and cultured cells, were identified by mass spectrometry (MALDI-TOFMS and CapLC-MS/MS) to be histones. Only a subset of tyrosine residues in histones was nitrated. For example, H4 was modified only at Tyr₉₈ and H2B only at Tyr₄₂ in tumors. Selective nitration may reflect differential accessibility of different tyrosine residues and the nearby molecules within the nucleosome. Prominent nitration of histones may reflect the relative stability of these modified proteins in tumors. This is the first use of mass spectrometry to identify nitrotyrosine-containing proteins modified *in vivo*. Nitration of histones *in vivo* may have biological significance.

5.2. Introduction

Reactive nitrogen oxide speices (NO_x) are nitric oxide ($\text{NO}^\bullet$)-derived species that are cytotoxic and mutagenic and have been implicated in the pathogenesis of several inflammatory disorders (25, 29, 36-41, 171). Inflammatory cells, such as macrophages, monocytes and neutrophils produce a relatively large amount of reactive NO_x . These species may exert deleterious effects by modifying or damaging various cellular targets including DNA, lipids and proteins (12, 13, 172). One of the targets in proteins is tyrosine, which can react with certain NO_x (e.g., ONOO^- , NO_2Cl) to form a fairly stable end-product, nitrotyrosine (27).



The presence of free nitrotyrosine amino acids or nitrotyrosine-containing proteins in biological samples is used as a molecular marker of reactive NO_x production in a tissue (27). Nitration of tyrosine residues in proteins may alter protein function (29-33), which may have both physiological and pathological significance. The presence of protein nitrotyrosine has been reported in inflammatory disorders such as *Helicobacter pylori* infection, Crohn's disease, ulcerative colitis, Wegener's granulomatosis, cystic fibrosis, asthma, obliterative bronchiolitis and rheumatoid arthritis (25, 29, 36-41). The level of protein nitrotyrosine has been found to correlate with the severity of inflammatory diseases (42-44). Protein nitrotyrosine has also been detected in some tumors (60-63). A high level of protein nitrotyrosine correlates with poor outcome in melanoma patients (64). Although a high level of protein nitrotyrosine has been detected *in vivo* in human or animal diseases, only a limited number of nitrotyrosine-containing proteins have been identified (173-181).

Several methods have been employed to detect and identify protein nitrotyrosine. Anti-nitrotyrosine antibodies permit ready detection by immunohistochemistry, Western

blotting or ELISA (24, 182). Typical methods for identification of specific nitrotyrosine-containing proteins involve immunoprecipitation of proteins with specific antibodies (173-178, 183-185). Immunoprecipitation has certain limitations; it is limited to proteins for which specific antibodies are available and it cannot identify which specific tyrosine residue(s) have been nitrated. Mass spectrometry of tryptic peptides is a sensitive and specific technique to identify proteins. Recently, it has been shown capable of identifying tryptic peptides containing nitrotyrosine and also specifying which tyrosine has been nitrated (30, 186). One recent report describes a technique of localizing nitrated proteins by Western blotting on a two-dimensional gel, followed by mass spectrometry, to identify putative nitrated proteins in tissue samples (8). However, positive identification of specific tyrosine residues nitrated *in vivo* has not previously been reported.

We have earlier shown that tumor-infiltrating neutrophils are capable of inducing genetic instability in Mutatect tumor cells *in vivo* (*chapter 3*) and have hypothesized that the neutrophils may induce this effect *via* releasing the mutagenic/cytotoxic NO_x into the microenvironment (*chapter 3* and *4*). Previous results from our laboratory suggest that tumor-infiltrating neutrophils are a source of reactive NO_x; this was demonstrated by immunohistochemical analysis of sections of tumors that showed that the neutrophils express inducible nitric oxide synthase. In addition, the tumor cells in the sections stained positively for nitrotyrosine by anti-nitrotyrosine antibody. The present chapter describes further analysis of nitrotyrosine-containing proteins in the tumors. Protein nitrotyrosine was detected using Western blot analysis, but mass spectrometry was also used to unambiguously identify some prominent nitrated proteins, including localization of specific tyrosine residues that have been nitrated. In addition, we examined whether an association between protein nitrotyrosine and genetic instability (*hprt* mutation frequency) exists.

5.3. Hypotheses

It was hypothesized that western blotting analysis would show the presence of nitrotyrosine in a wide range of proteins in Mutatect tumors. The tumors with high number of infiltrating neutrophils would have high level of protein nitrotyrosine.

5.4. Objectives and expected results

1. *To detect nitrotyrosine-containing protein(s) by western blot analysis in Mutatect cells growing in mice as tumors and in culture.* It was expected that nitrotyrosine-containing proteins will be higher in tumors than in culture since the former contain nitric oxide-producing neutrophils. A wide range of proteins, ranging in size from large to small molecular mass, were expected to contain nitrotyrosine.
2. *To compare the levels of protein nitrotyrosine as detected by western blotting with neutrophil content and hprt mutation frequency.* Since neutrophils are a source of nitric oxide and are capable of inducing mutations, the level of protein nitrotyrosine were expected to correlate with neutrophils and mutations.
3. *To induce formation of protein nitrotyrosine in cells growing in culture by exposing them to NO_x-donating compounds.* It was expected that the same pattern of nitrated proteins as found in tumors would be observed in cultured cells after exposure to NO_x donors.
4. *To identify any prominent nitrotyrosine-containing proteins in the tumors using mass spectrometry.*

5.5. Materials and Methods

Mutatect cell culture and tumor formation. All cell cultures were maintained in Dulbecco's modified Eagle's medium plus 10 % fetal calf serum (GIBCO-BRL, Burlington, Canada) in a humidified atmosphere of 5 % CO₂ at 37 °C. The properties of Mutatect TM-28 and TM-34 cells and tumor formation are described in *section 1.5*. All animal experiments were carried out at the Animal Resources facility of the Institute for Biological Sciences, National Research Council in Ottawa in accordance with guidelines of the Canadian Council on Animal Care.

Exposure of cells to NO_x generating compounds. The NO_x donors used were either glyceryl trinitrate (David Bull Laboratories, Canada) or sodium nitroprusside (Sigma-Aldrich Chemicals, St. Louis, MO). About 2×10^5 cells were seeded overnight in 10-cm dishes; the following day, an appropriate amount of NO_x donor was added to the culture media. For a 24 h exposure, 0.5 mM glyceryl trinitrate or 1.0 mM sodium nitroprusside was used; these

concentrations of the drugs reduced cell viability to about 70 % after 24 h, as determined by trypan-blue exclusion staining. For longer exposure to drug (>1 d), 0.1 mM sodium nitroprusside was used; viability was reduced to about 50% after 7 d. The culture medium was replaced with fresh drug every 3 d and cells were subcultured when they became confluent. At the end of an experiment, the plates were washed to remove floating dead cells and adherent cells were harvested after trypsin treatment. Harvested cells were ~90% viable, as determined by trypan-blue exclusion staining. These cells were used for protein extraction.

Protein samples. Cells were lysed in sodium dodecyl sulfate (SDS) sample buffer (2 % SDS, 10 % glycerol, 1 % 2-mercaptoethanol, 0.0005 % bromophenol blue, 125 mM MOPS, pH 6.8) and boiled for 5-10 min. For extraction of nuclei, cells were swelled in nuclei extraction buffer (10 mM Tris-HCl (pH 8.0), 85 mM KCl, 0.5 % Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride and 1 mg/ml aprotinin), incubated on ice for 20 min, and then homogenized with a tight-fitting Dounce homogenizer (Wheaton, Millville, NJ). Nuclei were collected by centrifugation at 400 ×g, resuspended in SDS sample buffer, sonicated and heated at 100°C for 5-10 min. Protein from tumors was isolated by homogenization and then sonication (in phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride and 1 mg/ml aprotinin); the homogenate was diluted in SDS sample buffer and heated at 100°C for 5-10 min. Fluorescamine was used to quantify protein (147). Nitrotyrosine-containing bovine serum albumin (BSA) was prepared by incubating BSA (6 mg/ml) in 10 mM NaNO₂, 9 μM FeCl₃, 0.3 % H₂O₂, and 20 mM sodium acetate (pH 5.6) for 24 h at room temperature.

Protein electrophoresis and western blot analysis. All protein extracts were resolved on a 12 % discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and either stained with Coomassie blue brilliant R-250 (Sigma-Aldrich) or electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore; Nepean, Canada) using a procedure that improves the transfer of basic proteins such as histones (*Appendix A2*). Membranes were stained reversibly with Ponceau S (Sigma-Aldrich) to detect the transferred proteins and the molecular weight standards. Where indicated, membranes were incubated with 20 mM Na₂S₂O₄ to chemically reduce nitrotyrosine residues (NO₂-Tyr) to aminotyrosine (NH₂-Tyr) (171). Membranes were washed twice in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 8), and then blocked with 2% BSA for 30–45 min. Anti-

nitrotyrosine rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY) was diluted at 1:2000 in TBST and incubated with the membranes for 1 h. Membranes were washed 4× in TBST and then incubated for 1 h with secondary antibody (alkaline phosphatase-linked goat anti-rabbit IgG; Kirkgaard & Perry Laboratories, Gaithersburg, MD) diluted 1:1000 in TBST. Membranes were washed 4× with TBST and then developed by using BCIP/NBT substrate (Sigma-Aldrich).

*Measurement of neutrophil content and *hprt* mutants in tumors.* The neutrophil content in tumors was estimated by quantifying the level of myeloperoxidase, a neutrophil-specific marker, as described in *sections 2.5*. The frequency of hypoxanthine phosphoribosyltransferase (*hprt*) mutants was measured in cells growing *ex vivo* as described in *section 3.5*.

Mass spectrometric analyses of proteins. This was carried out in collaboration with Dr. John F. Kelly of National Research Council of Canada. Protein extracts were first resolved by SDS-PAGE (20 µg/lane) and silver stained as previously described (187). The protein bands of ≤15 kDa were excised and in-gel-digested with modified trypsin (Promega, Madison, WI) without reduction/alkylation. The digested peptides were first analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) using a Voyager Elite STR (Applied Biosystems, Framingham, MA). The peptide mass fingerprints were used to search a non-redundant protein sequence database (NCBI) using MassFit™ (Protein Prospector™). Tryptic digests of ≤ 15 kDa proteins from SNP-exposed Mutatect nuclear extracts were analyzed by capillary liquid chromatography-tandem mass spectrometry (CapLC-MS/MS) using a hybrid quadrupole time-of-flight mass spectrometer (Q-TOFMS, Micromass, Manchester, UK). The extracts were resolved on a 0.3 mm × 15 cm PepMap C₁₈ capillary column (Dionex/LC-Packings, San Francisco, CA) using a gradient (5-95% acetonitrile 0.2% formic acid in 30 minutes, 3.5 µL/min) supplied by a CapLC HPLC pump (Waters Inc., Millford, MA). The mass spectrometer was set to operate in automatic MS/MS switching mode. MS/MS spectra were obtained only on doubly and triply protonated ions. These were then used to automatically search protein sequence databases using the manufacturer's database searching software (ProteinProbe™). Tryptic digests of ≤ 15 kDa proteins from Mutatect tumor extracts were analyzed by nanoelectrospray ionization-tandem mass spectrometry (nESI-MS/MS) using the Q-TOFMS.

Approximately one-third of the digest extracts were desalted using C₁₈ ZipTips™ (Millipore Inc., Bedford, MA). Desalted extracts were then loaded into gold-coated nanoelectrospray needles (Micromass). nESI-MS/MS analysis was carried out on all the peptide ions observed in each sample. Database searching was carried out as described above. Typically, modified peptides were not matched to any protein and were interpreted manually.

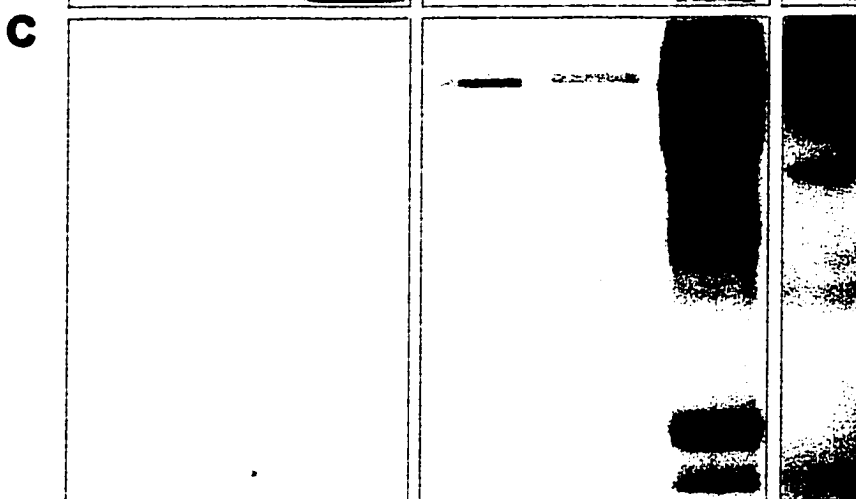
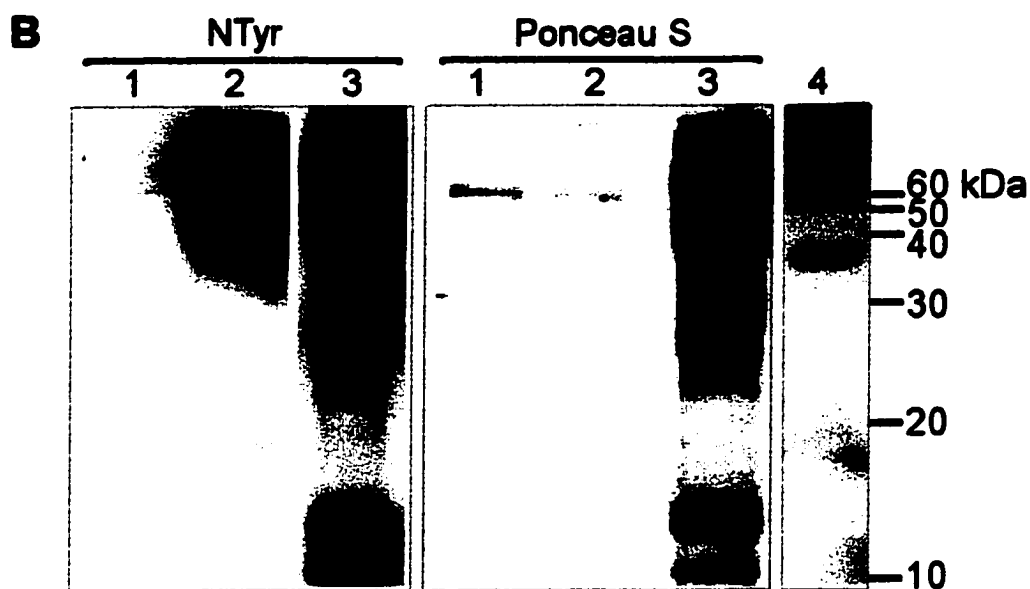
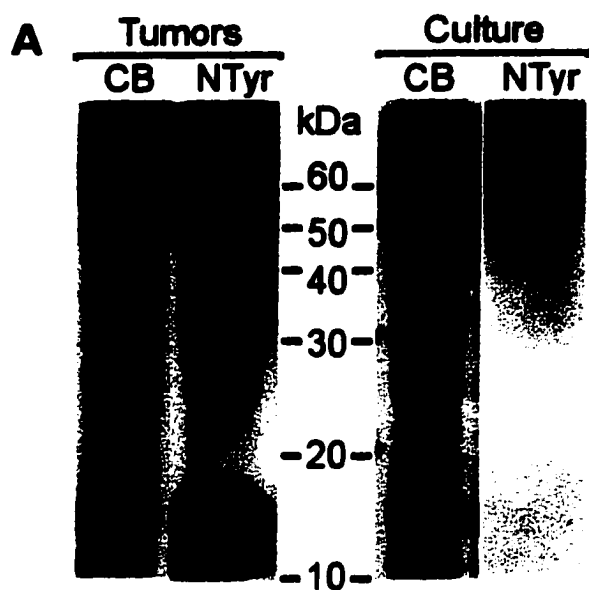
5.6. Results

5.6.1. Detection of nitrated proteins by western blot analysis in Mutatect tumor extracts

Previous results from our laboratory have reported on the presence of protein nitrotyrosine in Mutatect tumors by immunohistochemical analysis using an anti-nitrotyrosine antibody. Both cytoplasmic and nuclear staining in tumor cells was observed (70). To examine in more detail the nature of the nitrotyrosine-containing proteins, Mutatect tumor homogenates were analyzed by western blot analysis using an anti-nitrotyrosine antibody. A large number of nitrated proteins, ranging in size from large to small, were detected (Fig. 5.1A). The distribution of nitrotyrosine-containing proteins was similar, but not identical, to the distribution of protein detected by Coomassie blue staining. This suggested that some proteins were preferentially modified. The most prominent examples were ≤ 15 kDa proteins, presumed to be histones. These results were compared to extracts of cultured Mutatect cells. The difference between tumor extracts and cultured cell extracts was striking (Fig. 5.1A). In cultured cell extracts, there was only weak staining, limited to proteins of high molecular mass (> 50 kDa); no nitration of ≤ 15 -kDa proteins was detectable. The specificity of staining by the anti-nitrotyrosine antibody on western blots was demonstrated by (i) comparing chemically nitrated and unmodified bovine albumin (Fig. 5.1B, lane 1 and 2), (ii) absence of staining with secondary antibody alone (data not shown) and (iii) absence of staining after chemical reduction with Na₂S₂O₄ of nitrotyrosine to aminotyrosine residues (Fig. 5.1C, nitrotyrosine panel). The chemical reduction did not affect the antigenicity of an unrelated protein (thymidylate synthase in HeLa cell extracts), detected using a specific polyclonal antibody (188) (lanes 4 in Fig. 5.1B and C). These results indicate that a large number of proteins, including ≤ 15 -kDa proteins, are nitrated in subcutaneous Mutatect tumors in mice but not in cultured Mutatect cells.

FIGURE 5.1. Detection of protein nitrotyrosine (NTyr) in Mutatect tumors by western blot analysis using a specific anti-nitrotyrosine antibody.

A) Coomassie blue staining (CB) or western blotting (NTyr) of protein extracts (30 µg/lane) from either Mutatect TM-28 tumors or cultured TM-28 cells. **B)** Western blot analysis for NTyr and the subsequent Ponceau S staining of a blot containing 0.5 µg of BSA (lane 1), 0.5 µg of NTyr-containing BSA (lane 2) or 30 µg of protein extract from Mutatect TM-28 tumors (lane 3). Lane 4 is a HeLa extract (20 µg protein) probed with an anti-thymidylate synthase antibody (hTS-8.3). **C)** Same as in panel B, except that all the blots were treated with Na₂S₂O₄ prior to immunoblotting. Other details are described in experimental procedures.



5.6.2. Protein nitrotyrosine, neutrophil content and *hprt* mutations in Mutatect tumors

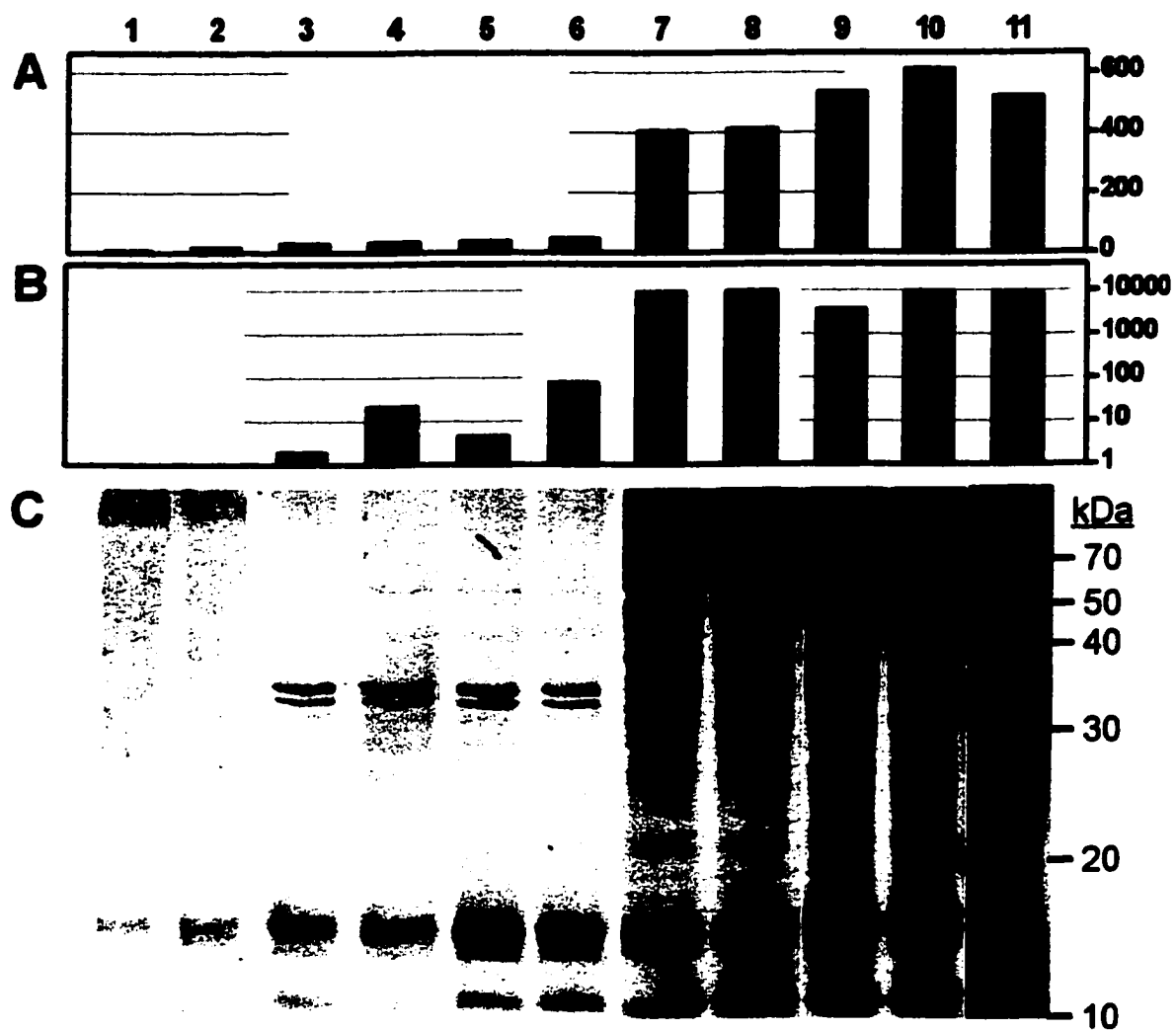
We have previously established that tumor-infiltrating neutrophils are the primary source of NO_x in Mutatect tumors and that the number of neutrophils correlates with the number of mutations arising at the *hprt* locus (70)(chapter 3). The level of neutrophil infiltration into Mutatect TM-34 tumors can be regulated by the administration of tetracycline, since TM-34 cells express a neutrophil chemokine (interleukin-8) from a tetracycline-responsive promoter (chapter 3). The neutrophil content (as measured by a myeloperoxidase, a neutrophil-specific marker) varied 16-fold as function of tetracycline added to the drinking water of tumor-bearing mice (Fig. 5.2A). Tumors from tetracycline-treated animals (lanes 1-6, Fig. 5.2A) had an average neutrophil content that was about 5% the level found in TM-34 tumors from animals receiving no tetracycline (bars 7-11, Fig. 5.2A). This difference in neutrophils, resulting from the down-regulation of interleukin-8 by tetracycline, was associated with a similar difference in the number of *hprt* mutants in the same two groups. The average number of mutants was 450-fold higher in the untreated compared to the tetracycline-treated tumor group (Fig. 5.2B; note the use of a log-scale). To provide evidence that neutrophil infiltration into tumors is associated with an increase in NO_x, we performed western blot analysis on tumor extracts using an anti-nitrotyrosine antibody. The number of nitrotyrosine-containing proteins differed greatly in tumor samples with a high neutrophil content compared to samples with a low content (Fig. 5.2C). Tumors with low neutrophil content had a low number of nitrotyrosine-containing proteins, which were predominantly ≤15kDa (Fig. 5.2C, lanes 1-6); nitration of protein bands at 32 and 34 kDa, consistent with positions for mouse histone H1, were also detected in some tumors. Conversely, tumors with high neutrophil content had a very high number of nitrotyrosine-containing proteins, which included proteins of both high and low molecular masses (Fig. 5.2C, lanes 7-11). These results are consistent with the notion that tumor-infiltrating neutrophils are the principal source of NO_x in Mutatect tumors.

5.6.3. Nitration of cellular proteins by exposure of cultured cells to NO_x donors

The data of Fig. 5.1A indicate that Mutatect cells, cultured under standard conditions, contain a relatively low level of nitrated proteins. Because a high number of nitrated proteins could be detected in Mutatect tumors but not in cultures (Fig. 5.1A), we tested

FIGURE 5.2. Neutrophil content, mutations and protein nitrotyrosine in subcutaneous Mutatect TM-34 tumors.

Each lane represents one tumor per mouse, and each mouse received 0.4 mM tetracycline (*lanes 1-6*) or no tetracycline (*lanes 7-11*) in drinking water. **A)** Activity of myeloperoxidase (a neutrophil-specific marker) in tumors. The activity is reported as units per mg protein. **B)** Mutation frequency (*hprt* mutants per 10^5 clonable cells) of the same tumors from panel *A*. **C)** Detection of nitrotyrosine-containing proteins in the same tumors (20 μ g of protein) from panel *A* by western blot analysis using an anti-nitrotyrosine antibody. Other details are described in Materials and methods.



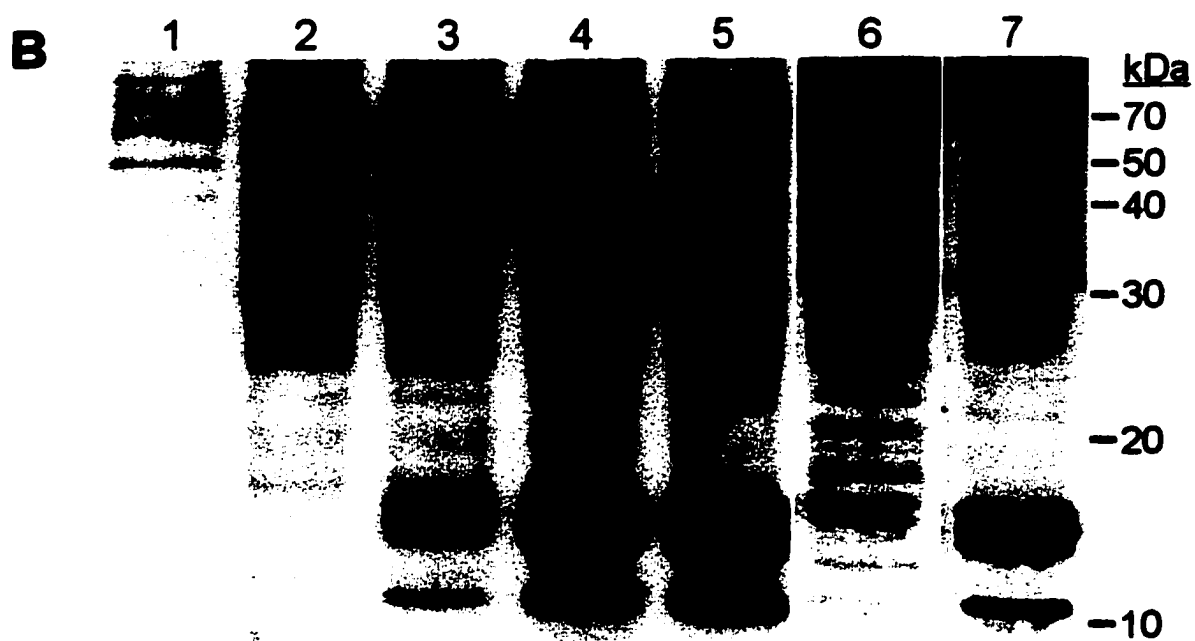
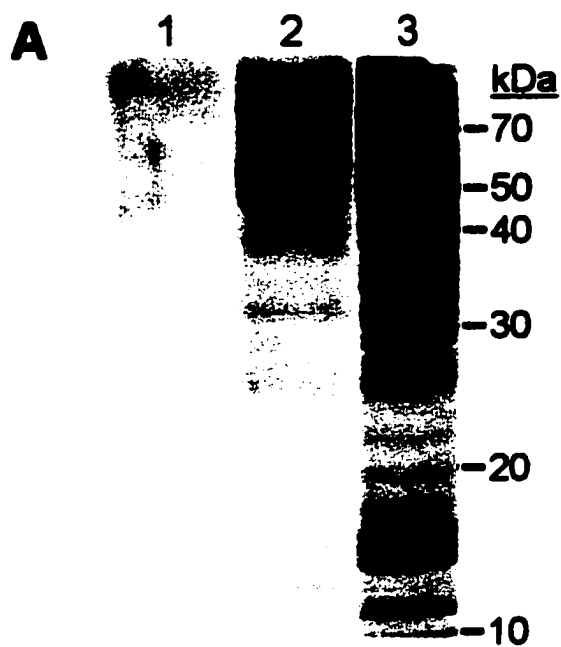
whether protein nitrotyrosine could be formed after exposure of cultured cells to a NO_x-donating drug. Mutatect TM-28 cells were cultured in the presence of sodium nitroprusside (SNP) and cell lysates were subsequently analyzed by western blotting using the anti-nitrotyrosine antibody. Exposure for 24 h to 1.0 mM SNP led to nitration of >40kDa proteins (Fig. 5.3A). Similar results were obtained after exposing cells for 24 h to 0.5 mM of glyceryl trinitrate (data not shown). However, in contrast to tumor extracts (Fig. 5.1 and 5.2), ≤15 kDa proteins were not nitrated. We therefore examined the possibility that a longer *in vitro* exposure (>24 h) to the drugs might mimic *in vivo* conditions, where cells are grown as subcutaneous tumors for 3 weeks. Cultured cells were therefore exposed for 0, 1, 3, 6 or 14 d to SNP. The concentration was reduced to 0.1 mM, a concentration that produced minimal effects on cell growth. Proteins from exposed cells were analyzed for the presence of nitrotyrosine modification by western blotting. After 3 d of 0.1 mM SNP exposure, nitration of ≤15 kDa proteins was observed, reaching an apparent maximum by day 6 with no further increase at day 14 (Fig. 5.3B). The level of nitrated ≤15 kDa proteins was comparable to the level found in tumors (Fig. 5.1A). Because we suspected that the ≤15 kDa proteins were histones, we separated 14 day-exposed cells into cytoplasmic (lane 6) and nuclear (lane 7) fractions and analyzed the nuclear fraction by western blotting. Essentially all of the nitrated ≤15 kDa proteins were found in the nuclear fraction (Fig. 5.3B, lanes 7). These experiments suggest that the ≤15 kDa proteins are likely to be histones and that they can be nitrated after extended (≥ 3 d) exposure to a NO_x-donating drug.

5.6.4. Identification of ≤15 kDa proteins and sites of modification by mass spectrometry

Strong nitration of ≤15 kDa proteins was observed by western blotting both in Mutatect tumors (Fig. 5.1 and 5.2C) and in cells exposed to NO_x-donating drugs for ≥3 d (Fig. 5.3B). To identify these proteins, a proteomics approach was used. Proteins from nuclei of Mutatect cell exposed to SNP for 14 d (Fig. 5.3B) were separated by SDS-PAGE and the 4 discrete ≤15 kDa proteins bands (Fig. 5.3C) were individually excised. Each was trypsin-digested and the resulting peptides analyzed by MALDI-TOFMS. The majority of tryptic peptides were products of core histones (H4, H2A, H2B and H4) (data not shown), confirming that the ≤15 kDa proteins were predominantly histones. Once this was

FIGURE 5.3. Nitration of proteins in cultured Mutatect cells after exposure to NO_x-donating compounds.

A) Western blot analysis for nitrotyrosine (*lanes* 1 and 2) and Coomassie blue staining (*lane* 3) of protein extracts (20 µg/lane) from Mutatect cells that were either not treated (*lane* 1) or treated with 1.0 mM of sodium nitroprusside for 24 h (*lanes* 2 and 3). **B)** Western blot analysis for the presence of nitrotyrosine in extracts (20 µg protein /lane) from Mutatect cells that were exposed to 0.1 mM of sodium nitroprusside for 0, 1, 3, 6 or 14 d (*lanes* 1-5, respectively). *Lanes* 6 and 7 are the western blot of the cytoplasmic and nuclear extract, respectively, of cells described in *lane* 5. **C)** Coomassie blue-stained <15 kDa proteins (from the nuclear fraction described in *lane* 7 of panel *B*). These were excised from the gel for mass spectrometric analysis. The positions of the predicted core histones are shown and these were confirmed by mass spectrometry. Other details are described in Materials and methods.



established, these peptides were analyzed by LC-MS/MS to determine whether any contained nitrotyrosine residues. At least 4 histone-derived peptides were found to contain nitrotyrosine residues (Table 5.1). In all cases, the non-nitrated counterpart of each nitrated peptide could also be identified (data not shown). Modification of tyrosine to nitrotyrosine was found only at specific sites: positions 72 and 98 in H4, positions 41 in H3 and positions 37, 40 and 42 in H2B. Other types of protein modification were also detected. Nitrosation of a histidine residue was observed in H4. Extensive oxidation of methionine and cysteine residues was observed in H4, H3, H2B and H2A (Table 5.1). These results indicate that exposure of cultured cells to NO_x-donating drugs can produce extensive modification of histones.

We also examined ≤15 kDa proteins present in tumor extracts (Fig. 5.2C). Extracts were separated by SDS-PAGE and the ≤15 kDa protein bands were analyzed as described above. These bands contained tryptic peptides from all 4 core histones (data not shown). The major modification identified in these tumor cell proteins was nitration of tyrosine residues at position 98 in H4 (Fig. 5.4) and position 42 in H2B (data not shown). Mass spectrometry has allowed us to positively identify that the majority of the ≤15 kDa proteins are histones. In addition, it has confirmed that nitrotyrosine is present in histones derived from both Mutatect tumors and cultured cells exposed to NO_x-donating drugs. For the first time in an *in vivo* model, we have shown that tyrosine modification was limited to specific sites on a protein.

5.6.5. Selectivity of nitration of tyrosine residues in the nucleosome

Selectivity of nitration of tyrosine residues has been previously reported in known proteins that were chemically modified by nitrating agents (189). These workers suggest that factors favoring tyrosine nitration include the following: (i) accessibility of the tyrosine residue to nitrating agents (ii) presence of the tyrosine residue in a loop structure formed by amino acid residues glycine or proline (iii) presence of the tyrosine in proximity to a negatively charged amino acid residue. In our study, nitration was restricted to Tyr₃₇, Tyr₄₀ and Tyr₄₂ in histone H2B, Tyr₇₂ and Tyr₉₈ in histone H4, and Tyr₄₁ in histone H3. Using Swiss-PdbViewer software, we examined whether this restriction was due to the factors described above (Table 5.2). The nucleosome structure shown in Fig. 5.5 highlights those

TABLE 5.1: Mass spectrometric determination of histone modifications in Mutatect cells exposed to 0.1 mM SNP for 14 days

Histones ^(a)	Sequence of the modified peptide ^(b)	Modification	
		Site	MW (Da) ^(c) Type
H4	T ₉₆ LYGFGG ₁₀₂	Y ₉₈	45 -NO ₂
	D ₆₈ AVTYTEHAK ₇₇	Y ₇₂	45 -NO ₂
	D ₆₈ AVTYTEHAK ₇₇	H ₇₅	30 -NO
	T ₇₉ VTAMDVVYALK ₉₁	M ₈₃	32 -O ₂
H3	Y ₄₁ RPGTVLR ₄₉	Y ₄₁	45 -NO ₂
	F ₈₄ QSSAVMALQEACEAYLVGLFEDTNLCAIHAK ₁₁₅	C ₁₁₀	48 -O ₃
	F ₈₄ QSSAVMALQEACEAYLVGLFEDTNLCAIHAK ₁₁₅	M ₉₀ or C ₉₆ and C ₁₁₀	64 -O and -O ₃ ^(d)
H2B	E ₃₅ SVSVVYK ₄₃	Y ₃₇ , Y ₄₀ , Y ₄₂ ^(e)	45 -NO ₂
	A ₅₈ MGIMNSFVNDIFER ₇₂	M ₅₉ and M ₆₂	64 -O ₂
	A ₅₈ MGIMNSFVNDIFER ₇₂	M ₅₉ and M ₆₂	32 -O
H2A	V ₄₃ GAGAPVYMAAVLEYLTAEILELAGNAAR ₇₁	M ₅₁	32 -O ₂
	V ₄₃ GAGAPVYMAAVLEYLTAEILELAGNAAR ₇₁	M ₅₁	16 -O

^(a) Nuclear extracts of the cells were run on SDS-PAGE (12%), detected by silver stain and the bands shown in Fig. 3C (corresponding to histones) were excised out and analyzed by CapLC-MS/MS after trypsin digestion.

^(b) The identified sequences correspond to a mouse H4 sequence (NCB accession # S03426), a mouse H3 isotype sequence (H3.1 with NCB accession # S06755), 2 possible mouse H2B isotype sequences (H2B.291A with NCB accession # P10853 or H2B.291B with NCB accession # P10854) and 3 possible mouse H2A isotype sequences (isotype H2A.O with NCB accession # P20670, isotype H2A(A).613 with NCB accession # AAB04768 or isotype H2A(B).613 with NCB accession # AAB04767). The first methionine of each histone is considered position # 0.

^(c) Calculated by subtracting the expected MW of the ion from the observed MW.

^(d) The -O is present on either M₉₀ or C₉₆, and the -O₃ is present on C₁₁₀.

^(e) Partial nitration of Y₃₇, Y₄₀ and Y₄₂

FIGURE 5.4. nESI-MS/MS analysis of the nitrated (top) and unmodified (bottom) histone H4 tryptic peptide, T₉₆₋₁₀₂.

The peptide was observed in the in-gel tryptic digests of the Mutatect tumor protein extract (lower band in Fig. 5.2C). The monoprotonated precursor ions were m/z 759.4 and 714.4 respectively. The b and y fragment ions are indicated in the MS/MS spectra. The peptide sequences are also provided together with those fragment ions that most clearly show the location of the modification (b_3 and y_5).

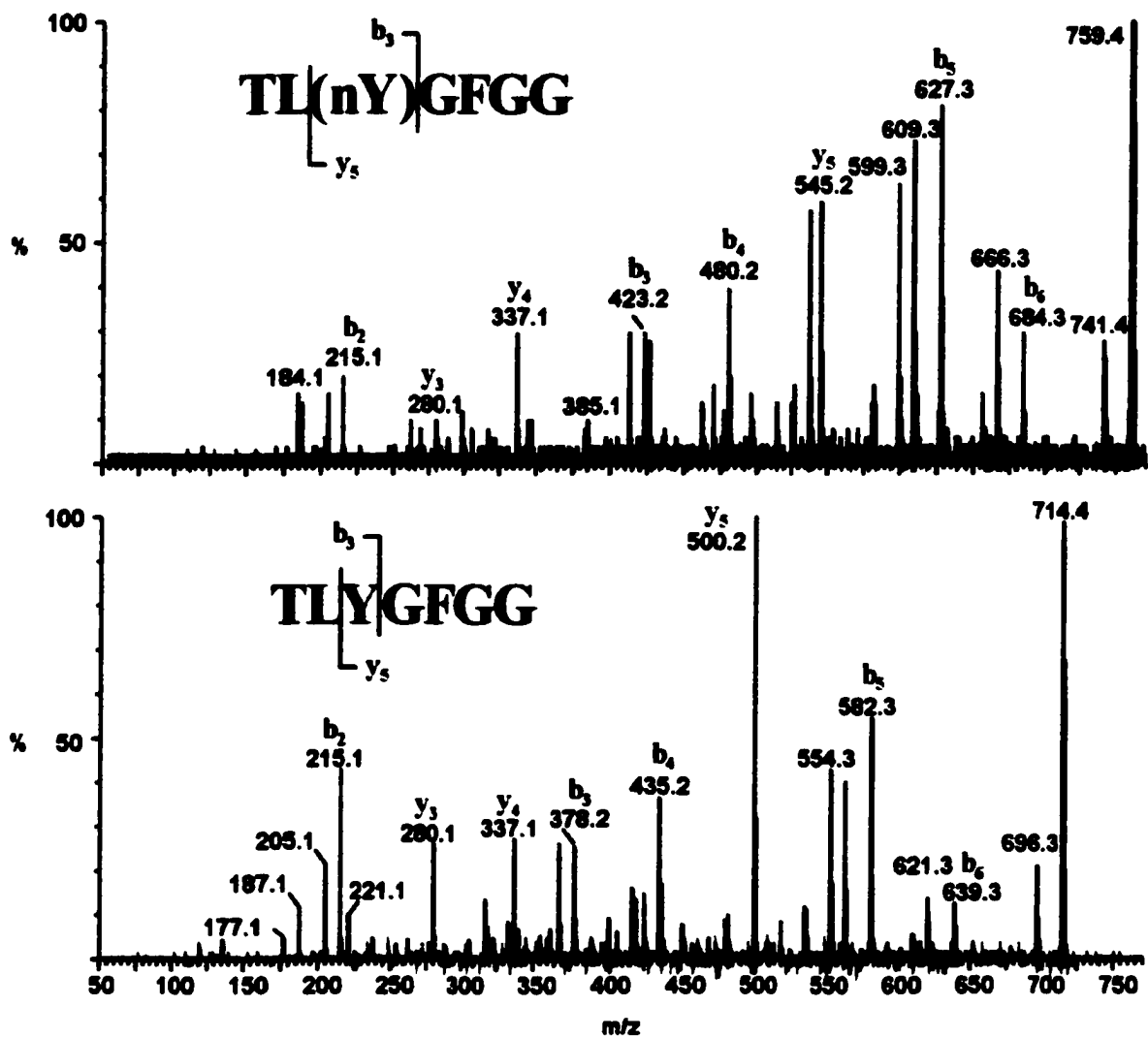


FIGURE 5.5. 3-Dimensional structure of a nucleosome.

Tyrosine residues that were found to be nitrated are highlighted in red and those found to be not nitrated are in blue. The structure is of *Gallus gallus* nucleosome particle (MMDB # 13235) and was drawn using Swiss-PdbViewer version 3.7b2 software (available at <http://www.expasy.ch/spdbv/mainpage.html>). The protein sequences of *Gallus gallus* and *Mus musculus* are >95% identical for histone H2A and H2B and >99% identical for H3 and H4. All tyrosine residues are conserved between the 2 species.



tyrosine residues that are nitrated and those that are not. The Tyr₄₂ of H2B was identified to be the most accessible of all tyrosine residues to the solvent. This site was nitrated both in cultured cells exposed to NO_x-donors and in Mutatect tumors. Nitration of other tyrosine residues, such as Tyr₉₈ (H4) and Tyr₄₁ (H3), may be facilitated by the fact that they are present in a loop in proximity to glycine or proline. Nitration of residues Tyr₇₂ (H4), Tyr₉₈ (H4) and Tyr₃₇ (H2B) may be facilitated by the fact that they are located in close proximity to negatively charged amino acids Asp₆₈ (H4), Asp₆₈ (H2B) and Glu₃₅ (H2B), respectively. Nitration of residues Tyr₄₀ (H2B), Tyr₄₂ (H2B) and Tyr₄₁ (H3) may be favored by their proximity to the negatively charged phosphate backbone of DNA in the nucleosome (Table 5.2). It has also been postulated that the presence of cysteine and methionine residues in the vicinity of tyrosine may eliminate interaction of tyrosine residues with the nitrating agents, since cysteine and methionine represent alternative targets for the nitrating agent (189). The absence of nitration of Tyr₈₇ (H4), Tyr₉₉ (H3) and Tyr₅₀ (H2A) may be attributable to the fact that they are in proximity to a cysteine or methionine (Table 5.2); this is supported by the observation that these cysteine or methionine were identified as being oxidized (Table 5.1). Thus, our observations support the notion that a combination of factors such as accessibility of tyrosine residues to the nitrating species, their position in the secondary structure (e.g., loop), and their proximities to cysteine, methionine or negatively charged molecules (e.g., aspartic acid, glutamic acid and DNA) may be responsible for the restricted nitration of tyrosine sites in histones.

TABLE 5.2: Factors affecting selectivity of tyrosine nitration in histones

Histones	Affected residue	Factors facilitating nitration ^(a)		Factors inhibiting nitration ^(a)
		Accessibility	Presence in a loop or nearby a Gly or Pro	
H4	Y ₇₂			4.0 Å from Asp ₆₈ (H4) 4.6 Å from Asp ₈₅ (H4) 6.0 Å from Glu ₇₆ (H2A)
	Y ₈₇			3.5 Å from Met ₈₃ (H4)
	Y ₉₈		In a loop and nearby several Gly	5.2 Å from Asp ₆₈ (H2B)
H3	Y ₄₁		In a loop and nearby a Pro and a Gly residue	5.0 Å from phosphate backbone of DNA
	Y ₉₉			4.0 Å from Cys ₉₆ (H3)
H2A	Y ₅₀			6.0 Å from Met ₅₁ (H2A)
H2B	Y ₃₇		In a loop	6.8 Å from Glu ₃₅ (H2B)
	Y ₄₀			3.6 Å from phosphate backbone of DNA
	Y ₄₂	Most accessible tyrosine in the nucleosome		4.8 Å from phosphate backbone of DNA

^(a) Distances between molecules were measured in the nucleosome particle of *Gallus gallus* (MMDB # 13235) (190) using Swiss-PdbViewer version 3.7b2 software (available at <http://www.expasy.ch/spdbv/mainpage.html>). The protein sequences of *Gallus gallus* and *Mus musculus* are >95% identical for histone H2A and H2B and >99% identical for H3 and H4. All tyrosine residues are conserved between the 2 species.

5.7. Discussion

Tyrosine nitration is a covalent posttranslational protein modification that occurs widely in association with chronic or recurrent inflammatory diseases (25, 29, 36-40) as well as in some tumors (60, 62, 63). The consequence to the disease processes of this type of modification has not been clearly established. However, when known proteins are chemically nitrated, this usually results in interference with their normal function (25, 30-33, 174) and an increase in proteasome-mediated degradation (191). Nitrotyrosine-containing proteins have been identified in various human and animal diseased tissues (Table 5.3). In most cases, nitrated proteins were identified by a combination of an anti-nitrotyrosine antibody and an available protein-specific antibody. In the case of manganese superoxide dismutase, N-terminal microsequencing was used to identify the nitrated protein (173). In the case of Mutatect tumors, the abundance and size of four low molecular weight ($\leq 15\text{kDa}$) proteins in nuclei led us to suspect that they were histones. Positive identification of all 4 core histones was possible using a combination of mass spectrometric techniques. These techniques have been useful in the past for studying pure proteins that have been chemically nitrated (30, 186, 192), but to our knowledge this is the first time that these techniques have been used to identify nitrated proteins in complex protein mixtures from tissue samples. This powerful methodology will no doubt see increasing application in the future for the identification of nitrated proteins.

The Mutatect tumor model has been useful for establishing a correlation between the number of tumor-infiltrating neutrophils and the number of mutated tumor cells (see *chapter 3*). Tumor-infiltrating neutrophils express inducible nitric oxide synthase and therefore are a source of potentially mutagenic NO_x . Protein nitrotyrosine has been shown to be present by immunohistochemistry in Mutatect tumors (70), where neutrophil-derived NO_x are likely responsible for the nitration. We have now confirmed by western blotting that Mutatect tumors contain nitrated proteins, whereas cultured Mutatect cells contain almost no nitrated proteins. Our ability to regulate the number of neutrophil infiltration into Mutatect tumors (*chapter 3*) has also allowed us to compare the level of nitrotyrosine-containing proteins in tumors with different neutrophil levels. Nitrated proteins were higher in tumors with a high number of neutrophils compared to low number. As expected from earlier studies, the number of neutrophils correlated well with the number of mutated tumor cells. These results

TABLE 5.3: Proteins known to be nitrated in humans or animal disease models

Nitrated protein(s)	Location	Reference
Mn-superoxide dismutase	Tubular epithelium of chronically rejected renal allografts	(173)
Prostacyclin synthase	Early stage atherosclerotic lesions in bovine	(174)
c-Src tyrosine kinase	Pancreatic adenocarcinoma	(175)
α-Synuclein	Brain lesions of neurodegenerative synucleinopathies	(179)
Ceruloplasmin		
Transferrin		
α1-Protease inhibitor	Plasma of acute respiratory distress syndrome	(180)
α1-Anti-chymotrypsin		
β-Chain fibrinogen		
Fibrinogen		
Transferrin	Plasma of lung cancer and smokers	(177)
Plasminogen		
Ceruloplasmin		
Myofibrillar creatine kinase	Rat model of heart failure	(176)
Surfactant protein A	Pulmonary edema fluid of acute lung injury	(178)
Histones	Mouse fibrosarcoma (Mutatect model)	This chapter

are consistent with the notion that tumor-infiltrating neutrophils are the principal source of mutagenic NO_x in Mutatect tumors.

Prominent nitration of histones was observed both in cultured cells exposed to NO_x-donating drugs for ≥3 d and in Mutatect tumors. In NO_x-exposed cultured cells, nitration of histones was not apparent until 3 d of exposure and then it increased with time, reaching a maximum at about 6 d. Interestingly, in the same cultures nitration of many cellular proteins >20 kDa was apparent after only 1 d of exposure and did not change very much with time (>6d). We speculate that the difference may be due to differences in accessibility of the different proteins to the nitrating NO_x. Nuclear proteins such as histones may be less accessible to the nitrating species than cytosolic proteins. In Mutatect tumors, on the other hand, the pattern of nitrated proteins was very different. In tumors with a low number of neutrophils, histones were nitrated just as in tumors with a high neutrophil number. The main difference between the two was that the >20kDa non-histone proteins were very heavily nitrated in the latter but not nitrated in the former. Histones are appreciably more stable than the average cellular protein and their slower turnover may permit them to accumulate nitrotyrosine more than high turnover proteins. Thus, the presence of nitrated histones in tissues may be a useful marker of long-term exposure to NO_x.

In unexposed cultured cells, small amount of protein nitration was sometimes observed especially in the high molecular weight proteins (see Fig. 5.3). It is postulated that this is due to low levels of nitric oxide produced by Mutatect cells. This is supported by the observation that cultured Mutatect cells appear to express a truncated iNOS protein as determined by western blot analysis (*J.K.Sandhu's PhD thesis*). However, nitrite accumulation is not detectable in the culture medium of the cells (data not shown). In addition, the same cells growing *in vivo* as tumors do not seem to express any form of iNOS as determined by western blot analysis and immunohistochemistry (*J.K.Sandhu's PhD thesis*).

Selectivity of nitration of tyrosine residues has been previously studied in chemically nitrated proteins (189). These researchers described several factors favoring tyrosine nitration including accessibility, proximity to a loop structure and proximity to a negatively charged amino acid residue. In our study, these factors may have been important in restricting the nitration of histones in the nucleosome to only a selectable number of

tyrosines. In addition, we postulate that the negative charge in the phosphate backbone of the DNA may also favor tyrosine nitration.

5.8. Significance

We have found a novel post-translational modification in histones *in vivo*, i.e., nitration of tyrosine residues. This modification was found to be only present in cells exposed to an inflammatory environment, containing cytotoxic and mutagenic NO_x. For the first time, mass spectrometric techniques were used to identify nitrotyrosine-containing proteins in biological tissues. We also show that this modification may in fact be a marker of long-term exposure to NO_x, which have been implicated in many inflammatory disorders including cancer and neurodegenerative disorders. Histones are the most abundant proteins associated with DNA in eukaryotes; post-translational modifications of histones can affect assembly of chromatin and expression of many important genes. Thus, in the research area of histone modification, this is a very significant finding since it warrants further investigation into the effect of the nitrotyrosine modification on chromatin assembly, gene expression and possible inhibition/activation of the other modifications. In addition, in the research area of inflammatory disorders and cancer, this is also a very significant finding. For years, many researchers have seen nuclear staining for nitrotyrosine in histological sections of various tissues containing inflammatory cells. Our finding that the nuclear staining likely represents histone modification suggests that those tissues were exposed for long-term to NO_x. In fact, in collaboration with a pathologist Dr. Susan Robertson at the Ottawa Hospital General Campus, our laboratory has recently translated this information into a clinical setting and found that nuclear nitrotyrosine staining is significantly higher in the prostate of prostate-cancer patients than control individuals.

5.9. Conclusions

Several nitrotyrosine-containing proteins were detected in Mutatect tumors. The level of nitration was higher in tumors containing high number of neutrophils than in tumors with low number. Mass spectrometric techniques have allowed us to add nitrotyrosine to the list of known post-translational modifications of histones that can occur *in vivo*. The fact that only a limited number of the tyrosine residues can be nitrated may be due to a

combination of factors, including the primary, secondary and tertiary structure of the nucleosome particle. The demonstration that nuclear proteins, in close proximity to DNA, can be nitrated is consistent with the evidence presented of NO_x-mediated DNA damage. Since nitrotyrosine can readily be detected immunohistochemically, the apparent stability of nitrated histones may make this protein modification useful as a nuclear marker of extended exposure of cells or tissues to NO_x.

Chapter 6. The role of glutathione in reactive NO_x-mediated mutagenicity in Mutatect cells

6.1. Chapter Summary

Reactive nitrogen oxide species (NO_x) are postulated to play a role in mutagenesis in Mutatect tumors (*chapters 3, 4 and 5*), but the mechanism of this mutagenicity is not well understood. Our laboratory previously demonstrated that the intracellular antioxidant glutathione is actually required for the induction of NO_x -mediated mutagenicity. It was postulated that glutathione reacts with NO_x to form *S*-nitrosoglutathione that in turn acts as a carrier of NO_x from cytoplasm to nucleus, liberating mutagenic species within the vicinity of DNA. In this chapter, we initially set out to study the effect of altering glutathione levels in Mutatect cells for their use in future animal experiments. Mutatect cell lines with high and low glutathione levels were developed by targeting the biosynthesis of glutathione. Effects of glutathione manipulation on growth rate and cell attachment were observed. Glutathione levels were also found to be protective against NO_x -mediated cytotoxicity. However, we could not demonstrate an induction of mutation using NO_x -donating drugs in Mutatect cells *in vitro*. Formation of *S*-nitrosoglutathione and other *S*-nitrosothiols were also not detectable in cells by these drugs. After testing several NO_x -donating drugs, two conditions were found to induce a large amount of intracellular *S*-nitrosothiol formation. Preliminary results show that the formation of intracellular *S*-nitrosothiols by these drugs may be associated with the induction of mutations in the tumor cells. However, intracellular *S*-nitrosoglutathione was not detectable under these conditions. Further examination showed the presence of unusually high levels of *S*-nitrosoglutathione-detoxifying activity in Mutatect cells growing in complete medium. We believe that the high levels of *S*-nitrosoglutathione-detoxifying activity in cultured Mutatect cells is responsible for the lack of induction of mutations by NO_x -donating drugs.

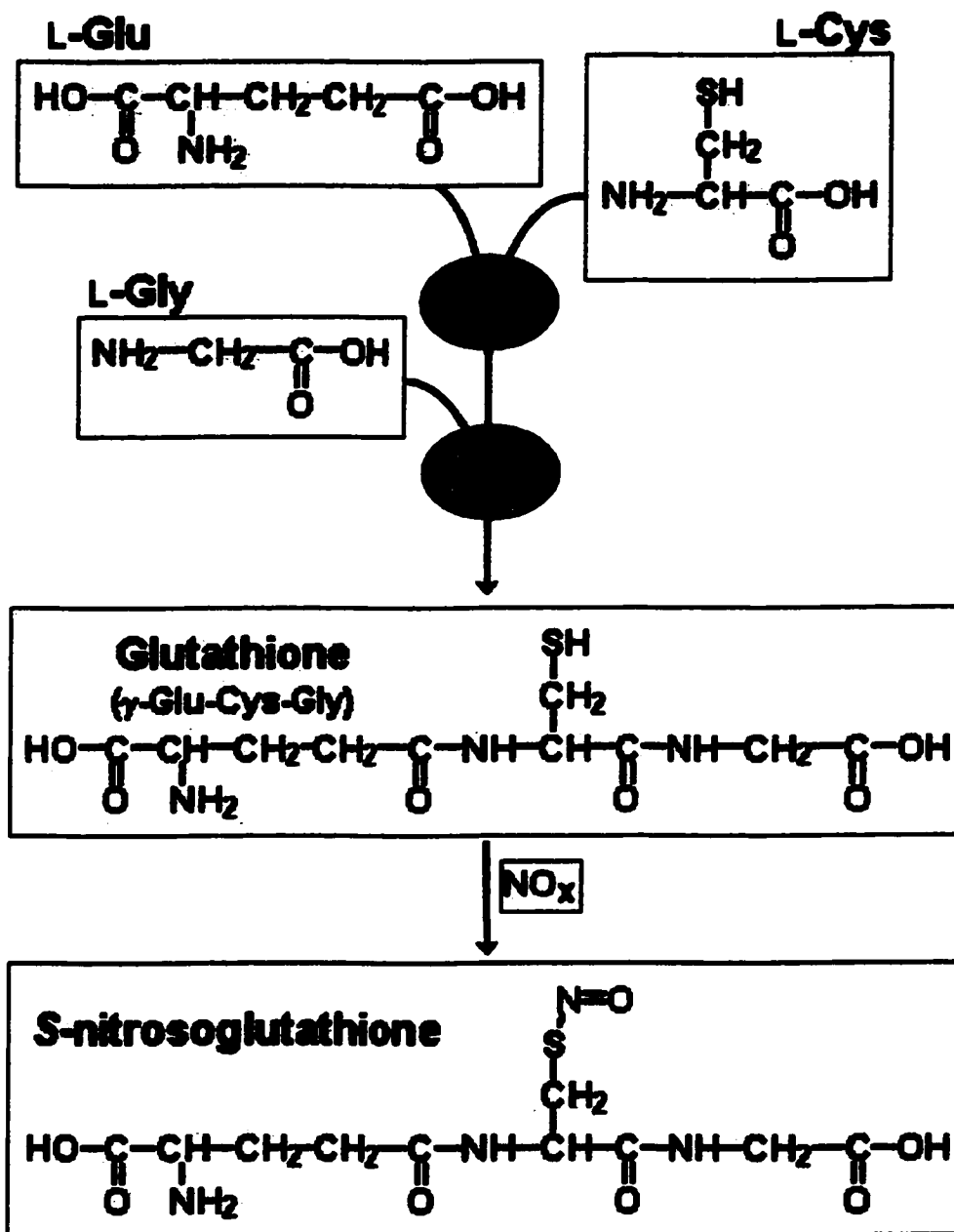
6.2. Introduction

Nitric oxide ($\text{NO}^\bullet$) and related species are collectively termed as reactive nitrogen oxide species (NO_x). Under physiological conditions, $\text{NO}^\bullet$ acts as a secondary messenger involved in the cGMP signalling cascade, vasodilation, and inhibition of platelet aggregation. However, chronic presence of $\text{NO}^\bullet$ and other NO_x has been implicated in the pathogenesis of many diseases, including cancer (25, 193, 194). These species are produced by a variety of cells, mainly inflammatory cells, and can have cytotoxic and mutagenic effects on target cells, which may include pathogens, cancer cells, normal fibroblasts, pancreatic and brain cells. Most cells, however, have protective mechanisms to combat against the reactive NO_x . The mechanism of protection against NO_x -mediated cytotoxicity is well studied. A key component of this protection is an intracellular antioxidant, glutathione. Many reactive NO_x are also mutagenic. It was earlier postulated that neutrophil-derived reactive NO_x species likely play a role in inducing mutagenicity (*hprt* mutations and interleukin-8 gene instability) in Mutatect tumors (chapters 3, 4 and 5). However, the mechanism of NO_x -mediated mutagenicity is not well understood.

Glutathione (GSH) is a tripeptide, consisting of L-glutamic acid, L-cysteine and L-glycine (Fig. 6.1). The active group of GSH is the thiol ($-\text{SH}$) of cysteine, which is a strong nucleophile (71). GSH is the major non-protein thiol-containing molecule in mammalian cells and is estimated to represent >90% of non-protein cellular sulfur. It is present in virtually all animal cells, often in relatively high concentrations (0.5-10 mM). The majority (>70%) of glutathione exists in the thiol-reduced (GSH) form; other forms include disulfide oxidized (GSSG) and mixed disulfide (GSSR). GSH can be derived from GSSG in an NADPH-dependent reaction catalyzed by glutathione reductase. However, the *de novo* synthesis of GSH occurs by the sequential action of 2 enzymes: γ -glutamylcysteine synthetase (γ -GCS) and GSH synthetase (Fig. 6.1). The reaction catalyzed by γ -GCS is the rate-limiting step in the biosynthesis of GSH and is a target of a specific and irreversible inhibitor, L-buthionine-(*S,R*)-sulfoximine (BSO). GSH is required for many crucial intracellular functions. One of its major functions is to protect cells from endogenous and exogenous electrophiles (xenobiotics and many drugs) by forming *S*-thiolated conjugates (71). These conjugates are either spontaneously formed or catalyzed by glutathione *S*-

FIGURE 6.1. Biosynthesis of glutathione and S-nitrosoglutatione.

De novo biosynthesis of glutathione from its constitutive amino acids, glutamate, cysteine and glycine, is catalyzed by rate-limiting γ -glutamylcysteine synthetase (γ -GCS) and glutathione synthase (GS). Reaction of glutathione with various reactive nitrogen oxide species (NO_x) readily generates S-nitrosoglutatione.



transferase and are eliminated from cells by various mechanisms. GSH is also a major intracellular antioxidant. It primarily functions as a direct scavenger of reactive nitrogen and oxygen species (such as NO_x , $\text{O}_2^{\cdot-}$, H_2O_2 , $\text{OH}^{\cdot}$) in enzyme-dependent or independent reactions (see *General Introduction*). Another major role of GSH is the maintenance of the redox balance and the essential thiol status of proteins by preventing oxidation of $-\text{SH}$ groups or by reducing disulfide bonds induced by oxidative stress (71). In addition, GSH acts as a storage for intracellular cysteine (71). Thus, as an abundant thiol-containing molecule, GSH plays a wide variety of important roles in cells.

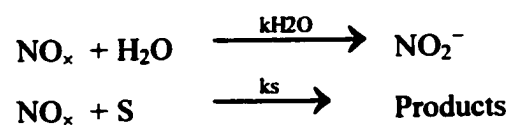
NO_x readily reacts with GSH and other thiol-containing molecules (e.g., protein and cysteine) to produce *S*-nitrosoglutathione (GSNO) (Fig. 6.1) and other *S*-nitrosothiols (RSNO). The selectivity of NO_x towards thiols is significantly higher than towards many other cellular molecules, such as protein tyrosine (Table 6.1). It is believed that, once inside the cells, NO_x predominantly reacts with thiol groups in GSH and protein to form GSNO and Pro-SNO. The RSNO are also capable of *releasing* different forms of NO_x (including $\text{NO}^{\cdot}$, NO^+ , NO^- , $\text{NO}_2^{\cdot}$ or NO_2^+) or promoting formation of N_2O_3 or ONOO^- (11, 195, 196). In fact, RSNOs are extensively used as NO_x -donating compounds in many *in vitro* experimental systems and have been shown to mimic the physiological effects of $\text{NO}^{\cdot}$, such as cGMP signaling cascade, vasodilatation and inhibition of platelet aggregation (196). The ability of thiols to readily react with and release NO_x has led to postulate by several workers and our laboratory that thiols (in GSH and protein) function as carriers of NO_x species (20, 77, 197). The NO_x -carrying property of glutathione (GSH), cysteine, hemoglobin and albumin has been postulated since their corresponding RSNO (GSNO, CYSNO, *S*-nitroso-hemoglobin and *S*-nitroso-albumin) have been detected and quantified *in vivo* (20, 89, 197). In fact, these RSNO may be responsible for some of the well-documented physiological processes that had been previously attributed to $\text{NO}^{\cdot}$ itself (19).

Reaction between GSH and NO_x is also important in *protecting* cells from cytotoxic levels of NO_x species. Depletion of cellular GSH (by BSO treatment) has been shown to sensitize many cells to killing by NO_x (72-76). It is believed that the GSH reacts with the NO_x species leading to the formation of the *less* reactive GSNO species. In addition, a mechanism of detoxification of GSNO in cells was recently described (90). Formaldehyde

TABLE 6.1: Substrate selectivity of reactive nitrogen oxide species in aqueous solution

Substrates	$k_s/k_{H_2O} (M^{-1})^{(a)}$
Glutathione	10 000
Cysteine	5 000
Tyrosine	25
Alanine and other amino acids	<5
Cytosine	0.005

^(a) From ref. (88). Rate constants were determined by Lineweaver-Burk plots of the following equations :



dehydrogenase (FDH), an enzyme originally known to detoxify cytotoxic and mutagenic formaldehyde in the cells (86, 87), is also capable of catalyzing the detoxification of GSNO in an NADH-dependent manner (90). Thus, the reaction between GSH and NO_x, leading to the formation of GSNO, plays several key roles in a cell.

The mechanism of NO_x-mediated mutagenicity is not well understood. Our laboratory was the first to demonstrate the involvement of GSH in NO_x-mediated mutagenicity using Mutatect cells *in vitro* (77). Mutatect cells were treated with BSO to deplete the intracellular GSH levels and then exposed to NO_x-donating compounds, sodium nitroprusside or glyceryl trinitrate, to examine the *hprt* mutation frequency. Although it was anticipated that the lack of GSH in cells would enhance the mutagenicity induced by NO_x drugs, the opposite effect was observed. Depletion of GSH by BSO largely *prevented* the induction of *hprt* mutations by NO_x-donating drugs. A trivial explanation that the lack of GSH prevented bioactivation of these drugs was ruled out by showing that it had little effect on the accumulation of nitrite (NO₂⁻), a stable byproduct and a surrogate of NO_x production (77). Thus, this suggested that GSH is actually required for the induction of NO_x-mediated mutagenicity. To explain this, it was postulated that GSH reacts with NO_x to form GSNO that in turn acts as a carrier of NO_x from cytoplasm to nucleus, liberating mutagenic species within the vicinity of DNA; in the absence of GSH, GSNO is not formed and mutations are not induced. Thus, one of the things we examined in this chapter is the formation of intracellular GSNO (and other RSNO) after exposure to NO_x-donating drugs.

Our laboratory previously attempted to demonstrate the role of GSH in mutagenicity in Mutatect tumors *in vivo*. The tumors were directly injected with BSO to deplete their GSH levels and analyzed for *hprt* mutation frequency *ex vivo*. However, ambiguous results were obtained, as some tumors showed high and some showed low mutation frequency⁶. This was attributed to the effects of BSO on cells other than Mutatect cells *in vivo*, such as neutrophils or blood vessels. For example, it has been shown that depletion of GSH levels affect cell surface proteins (198) that are believed to be important for the infiltration of neutrophils into Mutatect tumors (see section 4.7). A more reliable method would be to construct Mutatect cell lines in which GSH level is altered. In this chapter, description of the

⁶ Unpublished results from the Birnboim laboratory.

construction of Mutatect cells with permanently manipulated GSH is reported. Mutagenicity as well as cytotoxicity induced by NO_x-donors was also examined in these cell lines *in vitro*. The effects *in vivo* will be examined at a future time.

6.3. Hypotheses

The pro-mutagenic role of GSH involves the formation of intracellular GSNO, which acts as a carrier of NO_x in Mutatect cells. Cells with high levels of GSH will have high levels of NO_x-mediated mutagenicity while cells with low levels of GSH will have low levels of mutagenicity.

6.4. Initial objectives and expected results

The followings were the initial objectives we set out to examine in this chapter:

1. *To develop plasmids capable of expressing full-length sense or anti-sense mRNA of γ -GCS. It was expected that expression of the sense mRNA would increase the GSH levels, while expression of antisense mRNA would decrease the GSH levels.*
2. *To permanently manipulate GSH levels in Mutatect cells by stably transfecting γ -GCS sense- or antisense-expressing plasmids. Clones with high and low GSH levels were expected to be isolated.*
3. *To examine in vitro the cytotoxicity and mutagenicity induced by NO_x-donating compounds in cells with permanently manipulated GSH levels. Cells with high GSH levels were expected to be resistant to NO_x-mediated cytotoxicity, while cells with low GSH were expected to be sensitive. Conversely, cells with high GSH levels were expected to show high levels of NO_x-mediated mutagenicity while cells with low GSH levels were expected to show low levels.*
4. *To measure intracellular formation of GSNO and Pro-SNO after exposure to NO_x-donating compounds in Mutatect cells. GSNO and Pro-SNO were expected to be produced in cells.*

6.5. Materials and methods

Chemicals. L-Buthionine-(*S,R*)-sulfoximine (ICN Biomedicals Inc., Aurora, OH) was prepared immediately before use in PBS. Glyceryl trinitrate (5 mg/ml) was a pharmaceutical grade sterile solution in saline from David Bull Laboratories (Canada) Inc. Sodium nitroprusside (reagent grade) was from Sigma-Aldrich Chemicals (St. Louis, MO), and was prepared immediately before use in Dulbecco's modified Eagle medium (DMEM) plus 10% fetal calf serum. Reduced GSH, cysteine hydrochloride (CYSH), *N*-acetylcysteine (NAC) and *o*-phthalaldehyde (OPT) were from Sigma-Aldrich Chemicals. GSNO, CYSNO or SNAC were made by mixing equimolar concentration (0.1-1 M) of sodium nitrite with the respective thiols: GSH, CYSH or NAC. Reaction time was ~10 mins to generate ~75-95% of the product, which was kept in the dark at 0°C at all times. CYSNO was made fresh immediately before use, because of its instability. Other reagents were from Sigma-Aldrich, unless otherwise stated.

Construction of γ -GCS-expressing sense and antisense constructs. The sense construct hGCS/pCR3 (see *Appendix A1*) was a gift from Dr. Stewart (Ottawa Regional Cancer Centre). It contains the human full-length γ -GCS coding-region (lacking any untranslated regions) in the sense direction downstream of a cytomegalovirus promoter in a pCR3 mammalian expression vector (Invitrogen, Carlsbad, CA). For the antisense construct AS-GCS/pCR3 (see *Appendix A1*), an I.M.A.G.E. Consortium LLNL cDNA clone #1970400 was identified to contain mouse γ -GCS sequences and obtained from a commercial source, as described in *chapter 1*. the clone was digested completely with Xho I to isolate the insert (~2.5 kb) containing the full-length mouse γ -GCS cDNA. pCR3 was also digested with Xho I and ligated with the insert. A clone containing the cDNA under the cytomegalovirus in an antisense orientation by restriction nuclease analysis was identified.

GSH assay. The level of GSH was determined using *o*-phthalaldehyde. Washed cells were suspended in water and an equal volume of cold PCA (2 N perchloric acid) was added. After a 15-min incubation at 0°C, precipitated protein was removed by centrifugation. Perchloric acid was neutralized and removed by precipitation as potassium perchlorate with KOH. A fraction of the supernatant was diluted with 0.1 M potassium phosphate, 1 mM CDTA, pH 8.0, and reacted with 1/100 vol freshly prepared OPT reagent (1 mg/mL *o*-phthalaldehyde in methanol). After a 20-min incubation at room temperature, fluorescence was

measured (λ_{EX} 350 nm; λ_{EM} 5420 nm). Values are corrected for efficiency of recovery of GSH from cell extracts, estimated by adding a known amount of GSH to cells immediately before acid treatment.

Conditions for cell culture and transfections. See chapter 1 for details.

Exposure of cells to NO_x donors. Cells were “HAT” cleaned as described in chapter 3 to obtain a 100% *hprt*⁺ population. 3×10^5 cells were plated in 10-cm dishes and grown for 2 days to achieve a cell culture of $\sim 10^6$ cells. Each sample was done in triplicate. For testing with drug alone, 0.5 mM SNP or 0.5–1 mM GSNO was added for ~ 24 h. For testing with combination of drug, cells were pretreated with 1 mM SNP for ~ 24 h then treated with 0.5–1 mM GSNO, 1 mM SNAC, or 1 mM CSNO for 1–2 h.

Cytotoxicity measurements. After various treatments were completed, cells were removed by trypsin treatment and plated at 300 cells per 6-cm dish (in triplicate). After 9–10 day, colonies were counted after staining in Wright stain. Percentage survival was calculated as the number of colonies in treated cultures compared to untreated cultures.

Mutagenicity measurements. After treatment with NO_x donors, 3×10^5 cells were plated in 10-cm dish and cultured for 10–11 days to allow expression of the *hprt* mutant phenotype; the cells were subcultured every 3–4 days or when they became confluent. The cells were analyzed for *hprt* mutation frequency as described in chapter 3.

Measurements of NO[•]/NO_x released by and stability of NO_x-donors. One millimolar of each donor was added to DMEM +10% fetal calf serum or to PBS solution and the spontaneous NO[•] release was measured at 37°C under dark conditions using ISO-NO Mark II nitric oxide meter (World Precision Instruments Inc., Sarasota, FL). To measure the nitrite produced or the stability of the drug, 1 mM of each donor was added to DMEM +10% fetal calf serum and incubated in a humidified chamber containing 5% CO₂, 95% air at 37°C. For nitrite measurement, an aliquot was taken at different time points, diluted 5 $\times$ and analyzed for nitrite (see below). For measurement of drug stability, an aliquot was also taken at different time points, diluted 20 $\times$ in water and analyzed spectrophotometrically at 334 nm.

Nitrite and low MW RSNO measurements. For intracellular measurements, cells were washed 3 $\times$ in PBS, 1 $\times$ in saline and lysed by sonication in cold water. Proteins were precipitated out by adding sulfosalicylic acid (1.5% final) and centrifugation. Supernatants

were used for nitrite and low MW RSNO measurements using Saville-Greiss assay in 96-well plates. In duplicate wells, one labeled *A* and the other *B*, 50 μ l of the sample (e.g., protein-free supernatant) or standard GSNO (10-200 μ M) was added. To the respective wells, 50 μ l of solution A (0.1 % sulfanilamide in 0.5 N HCl) or solution B (0.2 % mercuric chloride in solution A) were added. After 5 min incubation at room temperature, 50 μ l of solution C (0.02% N-(1-naphthyl)-ethylenediamine dihydrochloride in 0.5 N HCl) was added to all the wells. The red-colored product made was analyzed after 5 min spectrophotometrically at 540 nm. Well *A* readings represent nitrite-only, while well *B* readings represent nitrite *plus* low MW RSNO. Each assay was repeated at least 2 $\times$ to obtain 3 independent readings.

Total RSNO and Pro-SNO measurements. For intracellular measurements, cells were washed 3 $\times$ in PBS, 1 $\times$ in saline and lysed by sonication in cold water. Lysate (50 μ l) was added to two tubes labeled *A* and *B*, one containing 50 μ l of solution A and the other containing 50 μ l of solution B. After 5 min incubation at room temperature, proteins were precipitated out by adding sulfosalicylic acid (1.5% final) and centrifugation. 100 μ l of each supernatant was transferred to 96-well plates, and 50 μ l of solution C was added to all the wells. The red-colored product made was analyzed after 5 min spectrophotometrically at 540 nm. Readings originating from *A* represent nitrite-only, while that from *B* represent nitrite *plus* total RSNO. Pro-SNO levels were obtained by subtracting the levels of low MW RSNO from total RSNO. Each assay was repeated >2-times to obtain 3 independent readings.

Protein measurements. Fluorescamine was used to quantify protein (147).

GSNO-detoxifying activity. Protein extracts in PBS from cells or tumors were analyzed for NADH-dependent GSNO-detoxifying activity. Fluorometric assay was used to monitor NADH consumption in the presence or absence of GSNO, as described elsewhere. Saville-Griess assay was used to monitor GSNO consumption in the presence or absence of NADH, as follows. 45 μ l of reaction buffer (0.2 mM GSNO, 0.5 mM CDTA, 20 mM Tris-HCl, pH 8.0) with or without NADH (0.2 mM) was added to 5 μ l of either BSA control (50 μ g) or protein extract (50 μ g). The reaction was incubated at 37°C for 5 min, at which time the reaction was stopped by addition of trichloroacetic acid (8.3%). The mixture was

immediately incubated on ice for ≥ 10 min and centrifuged. The supernatant was used to measure the GSNO and nitrite levels. Each reaction was carried out in triplicates. One enzymatic unit is defined as 1 μmol of GSNO metabolized per min.

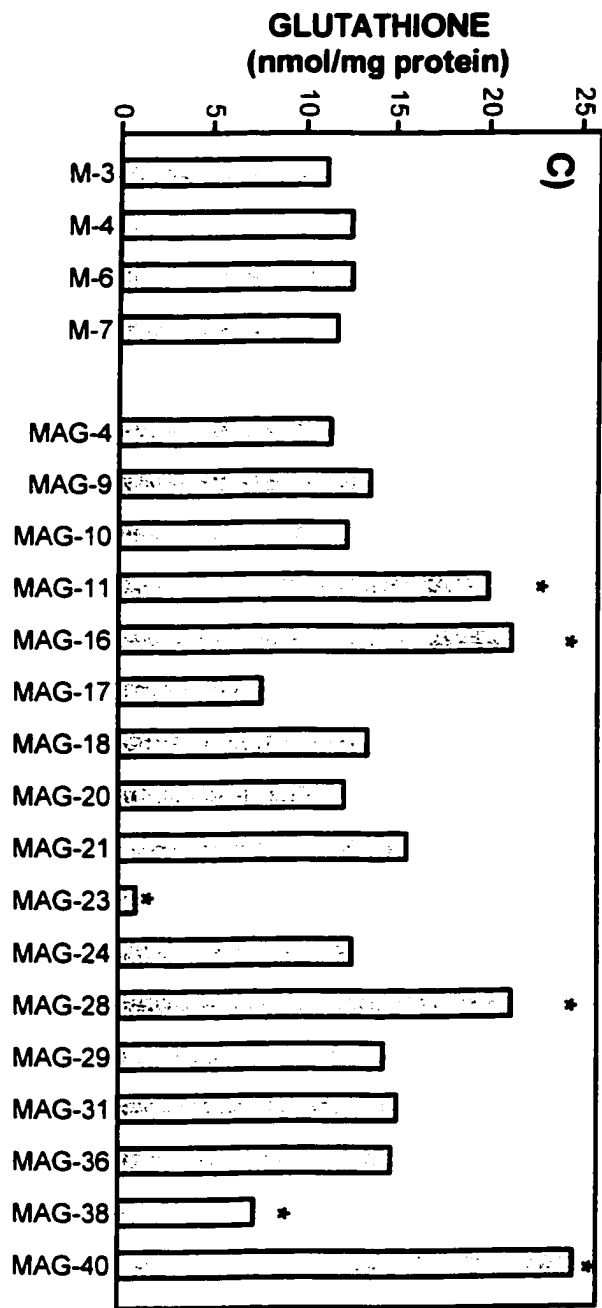
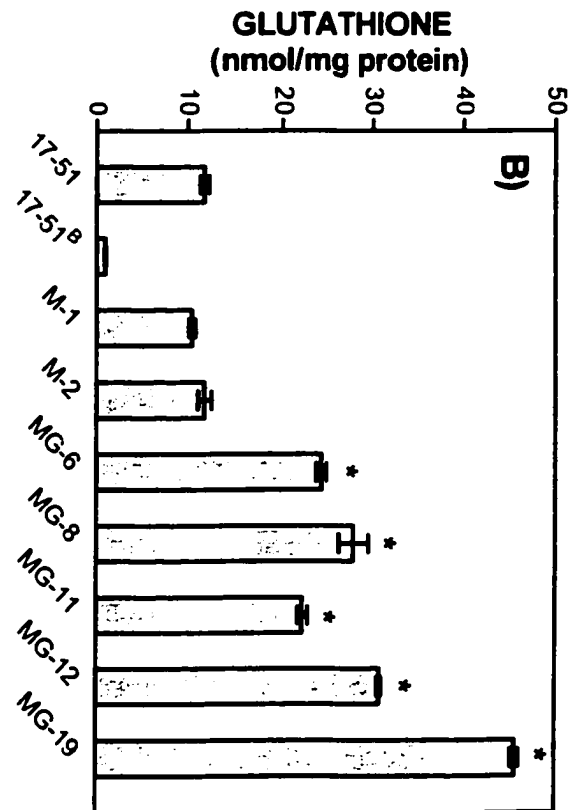
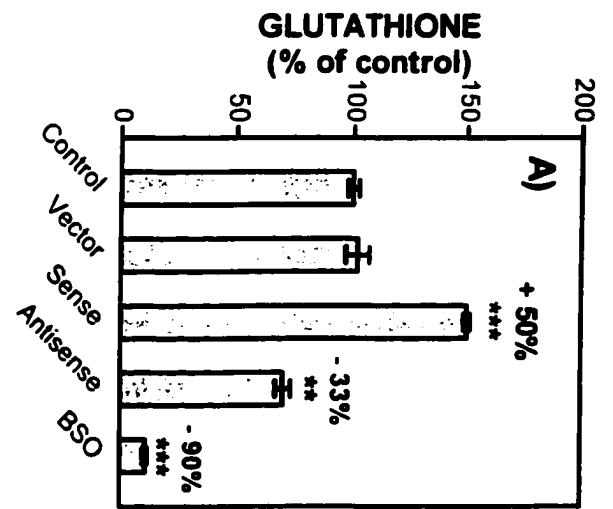
6.6. Results

6.6.1. Permanent manipulation of GSH levels in Mutatect cells

Development of Mutatect cell lines with permanently-manipulated GSH levels was accomplished by either over-expressing or under-expressing γ -glutamylcysteine synthetase (γ -GCS), the rate-limiting enzyme of the *de novo* biosynthesis of GSH. Constructs capable of expressing sense or antisense mRNA of γ -GCS were developed for over-expression or under-expression of γ -GCS, respectively. These constructs were first transiently transfected into HeLa cells to examine their ability to alter GSH levels (Fig. 6.2A). HeLa cells were used since they have significantly higher transfection efficiency than Mutatect cells (data not shown). As shown in Fig 6.2A, a construct expressing the sense mRNA of γ -GCS was able to increase the GSH levels by $\sim 50\%$, and a construct expressing the antisense mRNA of γ -GCS was able to decrease the GSH levels by $\sim 30\%$. Treatment with BSO, an irreversible inhibitor of γ -GCS, decreased the GSH levels by 90%. These constructs were then stably transfected into Mutatect MC17-51 cells. For controls, the cells were stably transfected with vector alone. On the basis of a linked neomycin resistant gene, the following types of clones were isolated: 19 sense-transfected *MG* clones, 40 antisense-transfected *MAG* clones, and 7 vector alone-transfected *M* clones (Fig. 6.2B and C). Five of the sense γ -GCS-expressing clones, MG-6, 8, 11, 12 and 19, were identified to have 2-to-4-fold higher GSH levels than either the control *M* clones or the parental MC17-51 cells (Fig 6.2B). Only one of the antisense γ -GCS-expressing clones MAG-23 had a very low level of GSH, 12 $\times$ lower than the controls (Fig 6.2C). Surprisingly, at least 4 of the antisense γ -GCS-expressing clones (MAG-11, 16, 28, 40) had significantly *higher* GSH levels than controls (Fig 6.2C). The mechanism of this is unknown. Thus, taken together these results show that GSH levels could be manipulated in Mutatect cells by over-expressing the sense or antisense mRNA of γ -GCS; clones MG-12 and MG-19 containing the highest level of GSH and clone MAG-23

FIGURE 6.2. Permanent manipulation of glutathione levels.

A) Demonstration of the ability of sense and antisense γ -GCS constructs to alter intracellular glutathione levels. HeLa cells were transiently transfected with sense or antisense constructs. **B)** Mutatect MG clones. Mutatect MC17-51 were stably transfected with the full-length sense γ -GCS construct and neomycin-resistant MG clones were isolated. 17-51^B are MC17-51 cells treated with BSO, an irreversible inhibitor of γ -GCS. **C)** Mutatect MAG clones. Mutatect MC17-51 were stably transfected with the full-length antisense γ -GCS construct and neomycin-resistant MG clones were isolated. Each bar represents mean $\pm$ SD of triplicate values. Other details are in Materials and methods.



containing the lowest level of GSH were used for further analyses, while M-7 and parental MC17-51 cells were used controls.

The effect of GSH manipulation on growth rate and cell attachment was observed. All cell lines with high levels of GSH (MG-12 and 19) had a similar growth rate as the controls, with doubling times of 14-16 h. However, it was noted that these cells were more adherent to the substrate. It took much longer to release them from the plastic Petri dish with trypsin than controls, suggesting that high GSH levels may cause these cells to adhere strongly to the culture plate. MAG-23 cells, which had very low levels of GSH, were found to grow significantly slower than controls, with a doubling time of ≥ 19 h. *N*-acetylcysteine (NAC), an intracellular deliverer of cysteine, was able to rescue the slow growth of these cells (Fig 6.3), but only temporarily (<1 week). In addition, MAG-23 cells appear to adhere very lightly to the culture plate, as they were easily released with trypsin compared to controls. These observations suggest that depletion of GSH is toxic for cells and that GSH can influence adherence of Mutatect cells to culture plates.

6.6.2. *NO_x-mediated cytotoxicity and mutagenicity in cells with altered GSH levels*

GSH is an intracellular antioxidant known to be a scavenger of NO_x species. Earlier studies from our laboratory have shown that NO_x-donating drugs, including SNP and GTN, are cytotoxic as well as mutagenic in Mutatect tumors (108). To examine whether NO_x-dependent cytotoxicity or mutagenicity would be affected in cell lines with manipulated GSH levels, Mutatect MG and MAG-23 clones were exposed to SNP for 24 h and analyzed for cytotoxicity and mutagenicity. In Mutatect MC17-51 and M-7 cells, SNP-induced cytotoxicity was ~40% (Fig 6.4). Compared to these control lines, the cytotoxicity was statistically significantly lower in MG-12 and MG-19 cells (10-25%) and higher in MAG-23 cells (~98%). Cytotoxicity of MAG-23 was comparable to that in BSO-treated MC17-51 cells (Fig 6.4). Similar results were obtained with another NO_x donor, glyceryl trinitrate (not shown). Thus, these results confirm that high levels of GSH are protective and low levels cause sensitivity to NO_x-mediated cytotoxicity in Mutatect cells.

To examine the effect of altered GSH levels on NO_x-mediated mutagenicity, the SNP-treated Mutatect cells were analyzed for *hprt* mutation frequency. MAG-23 cells could not be analyzed since they did not grow well (see above) and did not form colonies, which is

FIGURE 6.3. Growth curves of Mutatect M-7 and MAG-23 cells in the absence or presence of *N*-acetyl-cysteine.

MAG-23 are γ -GCS antisense-expressing cells while M-7 are control cells. One millimolar of *N*-acetyl-cysteine was used.

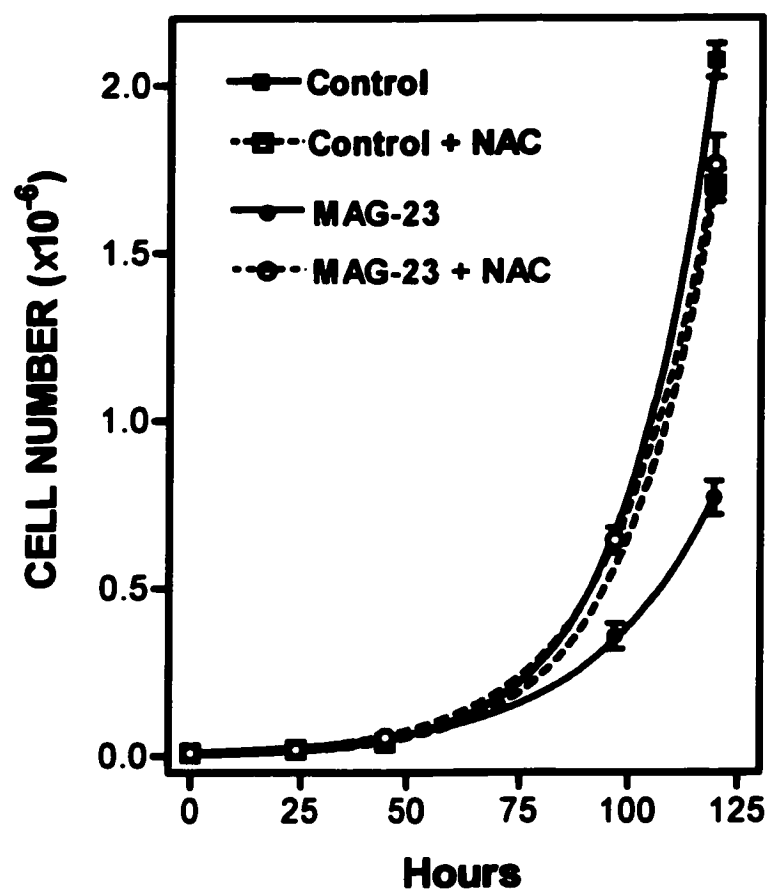
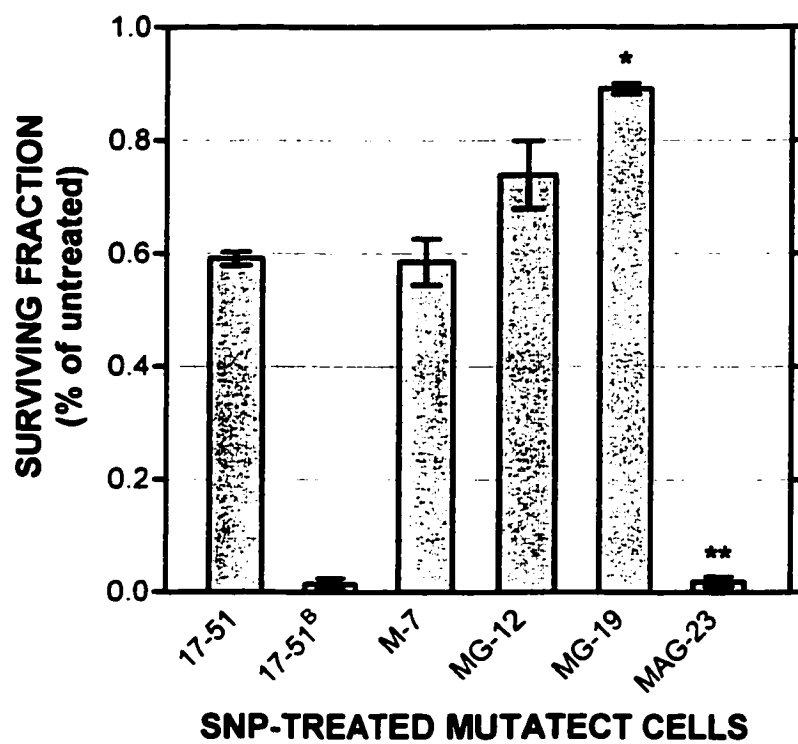


FIGURE 6.4. Cell survival following treatment with 0.75 mM SNP for 24 h.

17-51 are MC17-51 cells, while 17-51^B are BSO pretreated. Cell survival was measured using a clonogenic assay. Mean $\pm$ SD is shown.



necessary for measuring *hprt* mutations. Contrary to results published earlier (77, 108), our laboratory now finds that exposure to 1.0 mM SNP *in vitro* for 24 h is not able to induce *hprt* mutations in Mutatect cells. None of the Mutatect cells tested (MC17-51, M-1, MG-12, MG-19) showed any increase in mutation frequency after treatment with SNP (Fig 6.5A). Another donor, GTN was also unable to induce mutations. In addition, when different concentrations of SNP (0.1-1 mM) or GTN (0.2-0.7 mM) were tried and different time periods were tested (1-4 d), mutagenesis could not be induced *in vitro* (data not shown). Nor have we been able to induce mutations in Mutatect MN-11 cells (data not shown), which have been shown previously to be mutagenized by both SNP and GTN (108). These drugs were also not able to induce *hprt* mutations in Mutatect TM-28 cells. In contrast to these *in vitro* results, when these cells are grown as subcutaneous tumors, the mutation frequency is very high (Fig 6.5B). Thus, due to inability of NO_x-donating drugs to cause mutations *in vitro*, we were not able to examine the effect of NO_x-mediated mutagenicity in Mutatect cells with altered GSH state.

6.6.3. Are NO-donating compounds releasing NO or related species?

Since NO_x-donating compounds were unable to induce *hprt* mutations in Mutatect cells, their ability to spontaneously release NO[•] was examined. Besides SNP and GTN used above, the following compounds were also tested: *S*-nitrosoglutathione (GSNO), *S*-nitroso-*N*-acetyl cysteine (SNAC), *S*-nitrosocysteine (CYSNO) (Fig 6.6A). NO[•] released from the compounds was detected in cell-free culture media at 37 °C using *ISO-NO Mark II* nitric oxide meter. Release of NO[•] was detectable by all the RSNO tested (i.e., GSNO, SNAC, CYSNO) whereas no NO[•] was detected by SNP or GTN (data not shown). This is consistent with the known requirement of cellular metabolism to release NO[•] from SNP and GTN (196). Only after activation by light was SNP able to release NO[•] (data not shown). Another way to test whether these compounds were releasing NO_x in the presence of cells is to measure the accumulation of nitrite (NO₂⁻), one of the main end-products of auto-oxidation of various NO_x. As shown in Fig 6.6B and C, the rate of nitrite accumulation was very high by CYSNO, intermediate by SNAC and GSNO, and relatively low by GTN and SNP. This suggested that all the drugs are capable of releasing NO_x. Stability of GSNO, CYSNO and SNAC, as measured spectrophotometrically, can also be used as an indirect indicator of NO_x.

FIGURE 6.5. *Hprt* mutation frequency in Mutatetect cells after exposure to SNP and in cells recovered from tumors.

A) Mutation frequency in MC17-51, M-7, MG-12 and MG-19 cells untreated or treated with 1 mM SNP for 24 h. After 24 h, the medium was removed and replaced with fresh medium. After incubation for ~10 days to allow expression of the mutant phenotype, 6-thioguanine was added and mutant colonies were selected as described in Materials and methods. Each bar is a mean $\pm$ SEM of triplicate experiments. **B)** Mutation frequency in TM-28 cells exposed to SNP or cells recovered from tumors. Each bar is a mean $\pm$ SEM of triplicate plates or 5 animals.

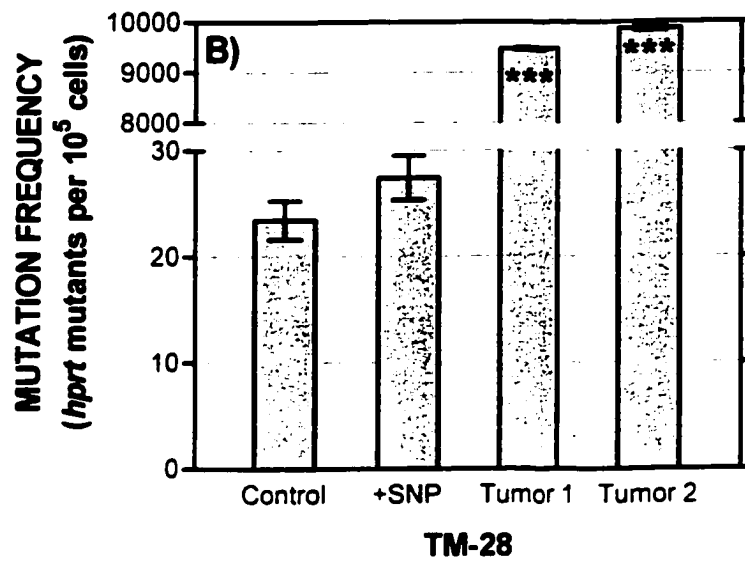
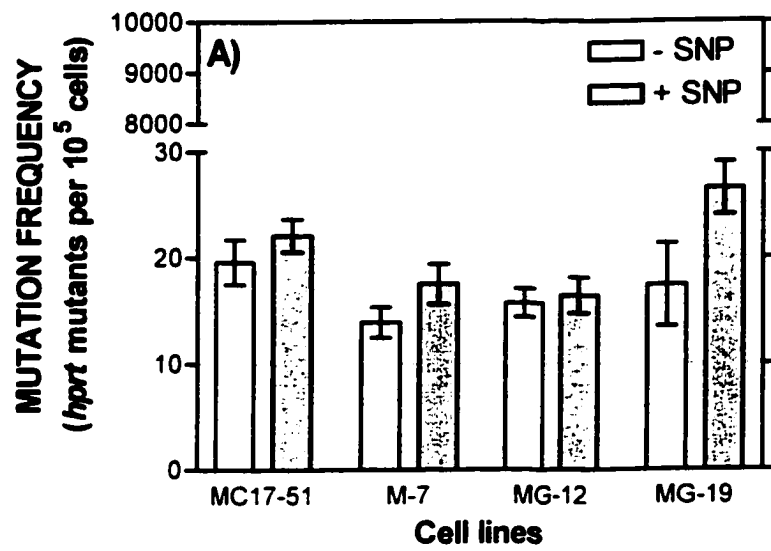
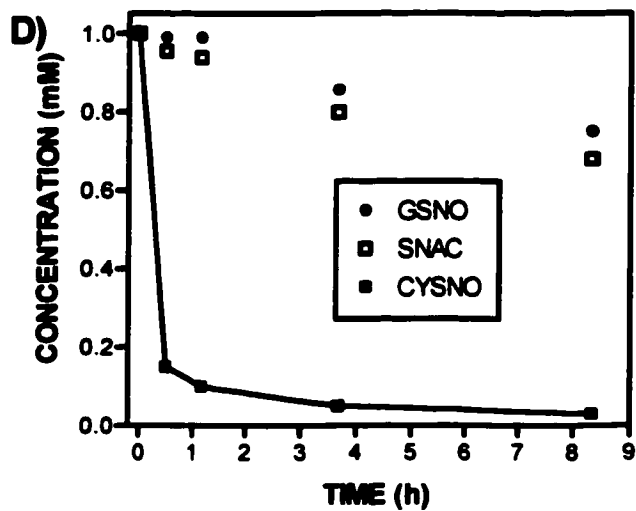
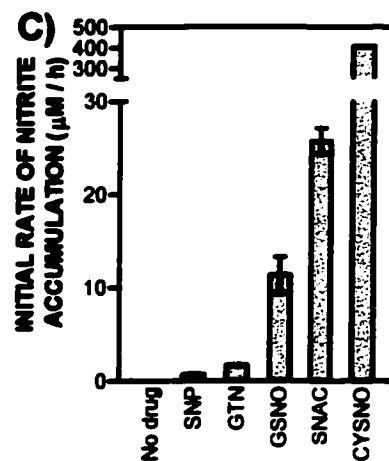
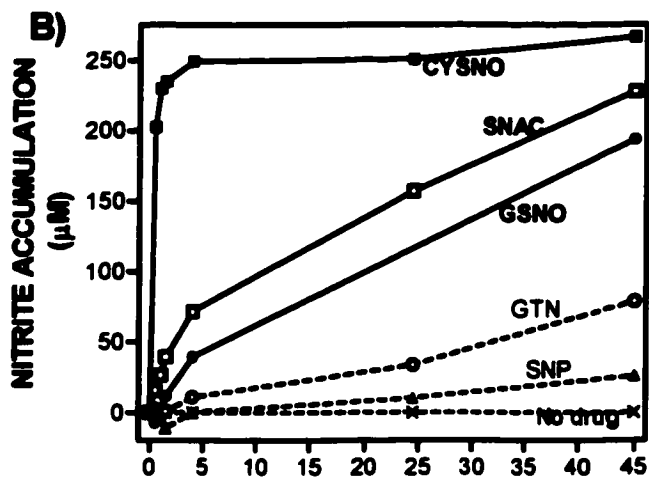
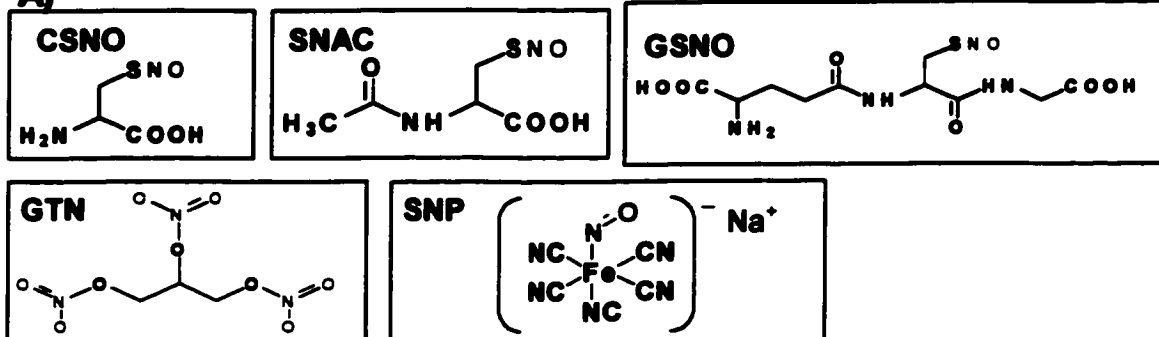


FIGURE 6.6. Properties of NO_x-donating drugs.

A) Structures of NO-donating drugs. **B)** Accumulation of nitrite by NO_x-donors. 1 mM of drugs were added to DMEM plus 10% fetal calf serum and incubated for the indicated amount of time at 37 °C. Nitrite was measured using Greiss assay. **C)** Initial rate of nitrite accumulation was estimated from the plots in panel *B*. **D)** Stability of the drugs. The stability was measured in DMEM plus 10% fetal calf serum in the absence of any chelators. Drugs were stable in the presence of chelators (e.g., DTPA; not shown). Experiments in panels *B* and *D* were carried out by Kiet (Skyz) Huong Do under my supervision.

A)



release. CYSNO was found to be significantly less stable than either SNAC or GSNO (Fig 6.6D), suggesting that the rate of NO_x release from RSNO is dependent upon their stability. Thus, although SNP and GTN do not spontaneously release NO[•], they are capable of releasing NO[•]-related species; the rate of release was as follows: CYSNO >> SNAC > GSNO > SNP > GTN.

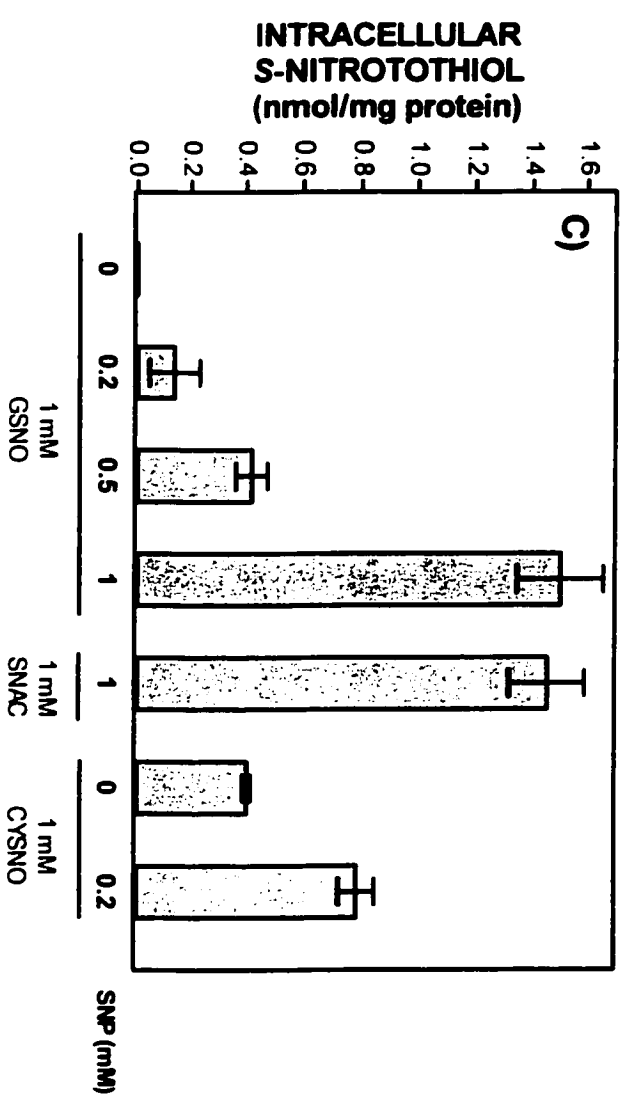
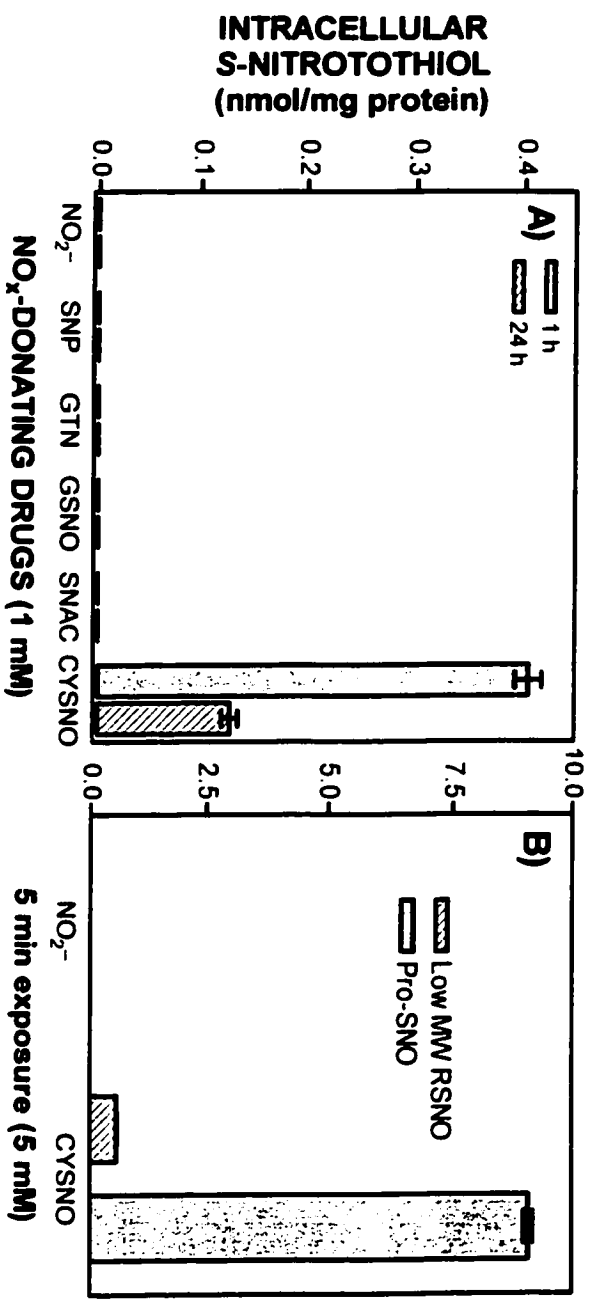
6.6.4. Intracellular RSNO formation after exposure to NO_x-donating compounds

Many NO_x species are known to readily react with free sulfhydryl groups (SH) to form RSNO. The various NO_x-donating compounds, described above, were therefore examined for their ability to induce RSNO formation in Mutatect cells. Cells were exposed to CYSNO, SNAC, GSNO, SNP or GTN for 1 h or 24 h and cell extracts analyzed for total RSNO levels using the Saville-Griess assay. RSNO could not be detected in the extracts after exposure to any of the compounds, with the exception of CYSNO (Fig 6.7A). This is consistent with the high-level production of NO[•]/NO_x by CYSNO (see above). To examine the type of the thiols that were S-nitrosated by CYSNO, extracts were fractionated into low-MW and high-MW (proteins) by acid precipitation and analyzed for RSNO; high-MW RSNO mainly represents Pro-SNO, while low-MW RSNO includes GSNO, CYSNO and other low MW species. Low-MW RSNO was not detectable in extracts of the CYSNO-exposed cells, suggesting that only Pro-SNO is formed (data not shown). Work by other researchers has shown that exposure of cells to higher concentrations of CYSNO (5 mM) for 5-10 min leads to a large amount of RSNO formation in cells (199). When these conditions were tested in Mutatect cells, a large amount of Pro-SNO and some low-MW RSNO was detectable (Fig 6.7B). The small amount of low-MW RSNO was likely the CYSNO and not GSNO since it was also observed in cells treated with BSO, a GSH-depleting drug (see chapter 7).

Various *combinations* of the NO_x-donating compounds were also tested for their ability to induce RSNO formation in Mutatect cells. It was found that either SNAC or GSNO were able to induce a large amount of Pro-SNO formation, but only after *pretreatment* of Mutatect cells with SNP for >16 h (Fig 6.7C). The level of RSNO formation was dependent upon the concentration of SNP (0.2-1.0 mM) used for the pretreatment (Fig 6.7C). Low-MW RSNO formation was again not observed under these conditions (not

FIGURE 6.7. Intracellular RSNO formation after exposure to NO_x-donating drugs.

A) Mutatect cells exposed to 1 mM of nitrite or each drug for 24 or 1 h. Cells were analyzed for total RSNO. **B)** Mutatect cells exposed to 5 mM of nitrite or CYSNO for 5 min. Cell extracts were fractionated into low and high MW RSNO and analyzed. **C)** Mutatect cells exposed to a combination of SNP and GSNO, SNAC or CYSNO and analyzed for total RSNO. Cells were first pretreated with the indicated concentration of SNP for 18 h and then 1 mM GSNO, SNAC or CYSNO was added for 1 h. Saville-Greiss assay was used to quantify all the intracellular RSNO. Each bar represents mean $\pm$ SEM of triplicate values. Other details in Materials and methods.



shown). SNP-pretreatment also enhanced the Pro-SNO formation by CYSNO (data not shown). The mechanism of the synergy of SNP-pretreatment on RSNO formation is unknown. Pretreatment by other drugs, such as GTN, did not induce any RSNO formation by GSNO or SNAC in the cells (data not shown). In addition, neither GTN nor SNP were able to induce RSNO formation after SNP-pretreatment. Thus, of all the compounds tested, only CYSNO was able to induce Pro-SNO formation on its own, while SNP-pretreatment was required for SNAC or GSNO to induce Pro-SNO formation; intracellular GSNO was not detected under these conditions.

6.6.5. NO_x -mediated cytotoxicity and mutagenicity in cells containing *S*-nitrosothiols

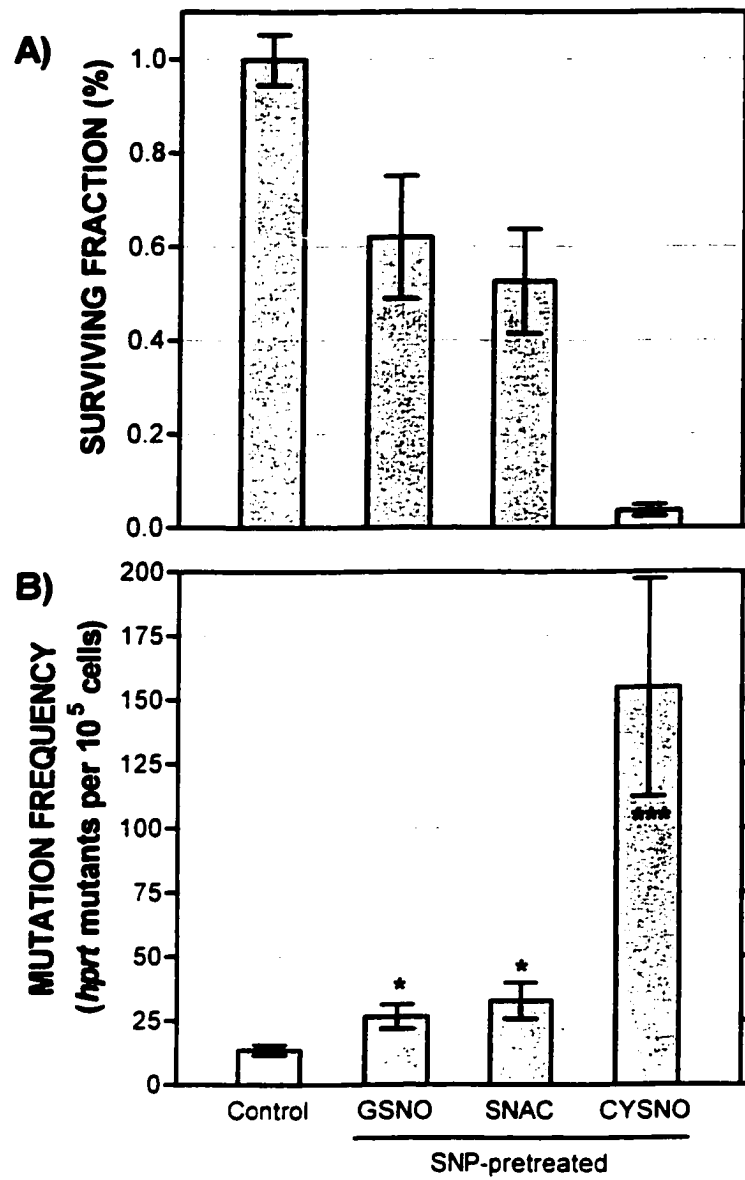
Although both SNP and GTN were able to induce cytotoxicity, neither was able to induce mutagenicity in Mutatect MC17-51 cells (see 6.6.2). However, as described above, these drugs were also not able to induce RSNO formation in the cells. We therefore examined whether conditions that did lead to intracellular RSNO formation could also induce *hprt* mutations. As demonstrated above, intracellular RSNO could be detected in extracts of cells that were first pretreated with 0.2-1 mM SNP for >16 h and then exposed to 1 mM of CYSNO, SNAC or GSNO for 2h. These cells were analyzed for cytotoxicity and mutagenicity. Both GSNO and SNAC were found to induce 40-50% cytotoxicity in SNP-pretreated cells, whereas CYSNO induced a significantly higher level of cytotoxicity (~95%) (Fig. 6.8A). The mutagenicity was found to be similar to the cytotoxicity. *Hprt* mutation frequency was about 2× higher in cells that were exposed to GSNO or SNAC after SNP-pretreatment than in cells that were not exposed to any NO_x -donating drugs (Fig. 6.8B); the difference was only weakly statistically significant ($P < 0.05$). On the other hand, the mutation frequency was about 10× higher in CYSNO/SNP-exposed cells than in untreated cells (Fig. 6.8B); the difference was statistically significant ($P < 0.001$). Thus, these results show that conditions that lead to high intracellular RSNO formation might also lead to high cytotoxicity and mutagenicity in Mutatect cells.

6.6.6. *S*-nitrosoglutathione-detoxifying activity in Mutatect cells

Intracellular GSNO was not detectable under the same conditions where to intracellular Pro-SNO could be detected (see 6.6.4). It has recently been shown that

FIGURE 6.8. Cell survival (A) and hprt mutation frequency (B) in Mutatect MC17-51 cells after exposure to a combination of SNP and GSNO, SNAC or CYSNO.

Cells were first treated with 1 mM SNP and then treated with 1 mM GSNO or SNAC for 1 h. In the case of CYSNO, 0.5 mM SNP was used for pre-treatment (to prevent cytotoxicity by CYSNO) and then 1 mM CYSNO was added for 1 h. Each bar represents a mean $\pm$ SEM of triplicate plates. Other details are described in Materials and methods.



formaldehyde dehydrogenase is able to detoxify GSNO in the presence of NADH but is unable to detoxify Pro-SNO. We therefore examined whether the NADH-dependent GSNO-detoxifying activity is also present in protein extracts of Mutatect cells. Consumption of NADH by the cell extracts in the absence or presence of GSNO was first monitored fluorometrically (Fig. 6.9A). As expected, cell extracts were able to consume NADH in the absence of GSNO, which was likely due to other NADH dehydrogenases present in the cells. The consumption was significantly enhanced in the presence of GSNO, suggesting that GSNO-detoxifying activity is likely present in these cells. To confirm this, consumption of GSNO by cell extracts in the absence or presence of NADH was monitored using the Saville-Griess assay (Fig. 6.9B). In the absence of NADH, GSNO was slightly consumed over a 6-min exposure to cell extracts. However, in the presence of NADH, GSNO was very rapidly consumed by the extracts. The specific activity in the extracts was 0.06 Units/mg protein. When the extracts were heat-inactivated, they were unable to catalyze GSNO-detoxification (Fig. 6.10A). The detoxifying activity was found to be specific to GSNO, since cell extracts were unable to detoxify Pro-SNO or CYSNO. Thus, these results show that Mutatect cells are capable of detoxifying GSNO but not Pro-SNO or CYSNO.

The level of GSNO-detoxifying activity in Mutatect cells was also compared with that of other cells and conditions (Fig. 6.10B). HeLa and NT-2 cell lines had ~50% lower activity than Mutatect cells. In addition, the activity in Mutatect tumors was found to be ~24% lower than the activity in Mutatect cultured cells. Finally, when Mutatect cells were cultured in a serum-free medium, the GSNO-detoxifying activity was reduced by ~30%. I conclude that Mutatect cells growing in culture in complete medium to have a high level of GSNO-detoxifying activity.

FIGURE 6.9. GSNO-detoxifying activity in Mutatect cells.

A) Consumption of NADH by cell extracts in the absence or presence of GSNO. GSNO (20 nmol) and/or protein extract (50 μ g) was added to 25 nmol NADH in 20 mM Tris-HCl (pH 8), 0.5 mM CDTA and the fluorescence was monitored (Ex_{340} , Em_{455}). **Inset:** GSNO-dependent NADH consumption in the absence (dotted line; curve $b - a$) or presence of cell extract (solid line; curve $d - c$). **B)** Consumption of GSNO by cell extracts in the absence or presence of NADH. GSNO was monitored by the Saville-Greiss assay. Other details in materials and methods.

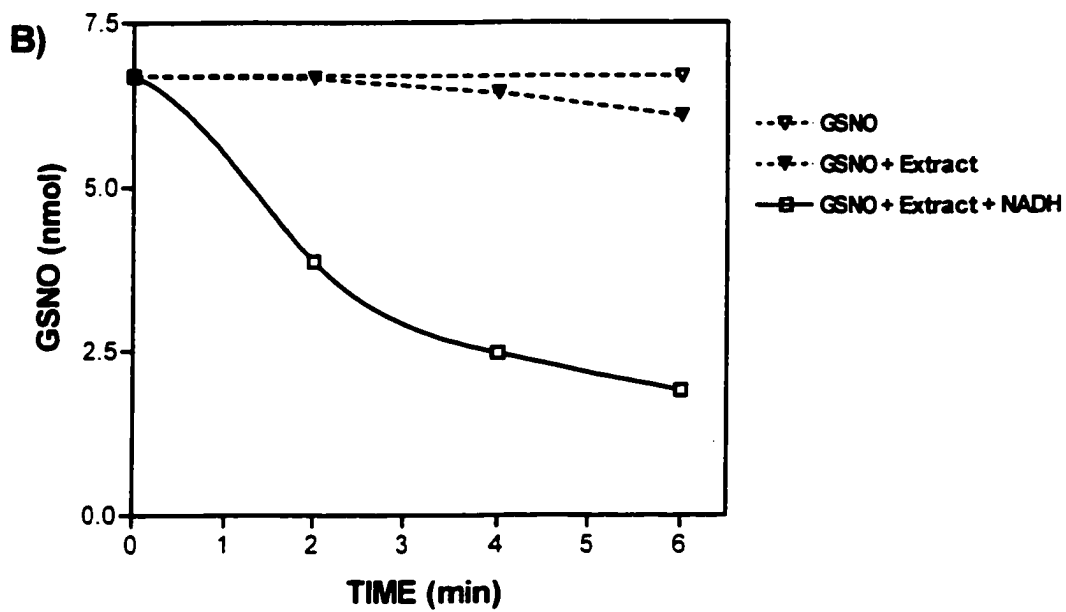
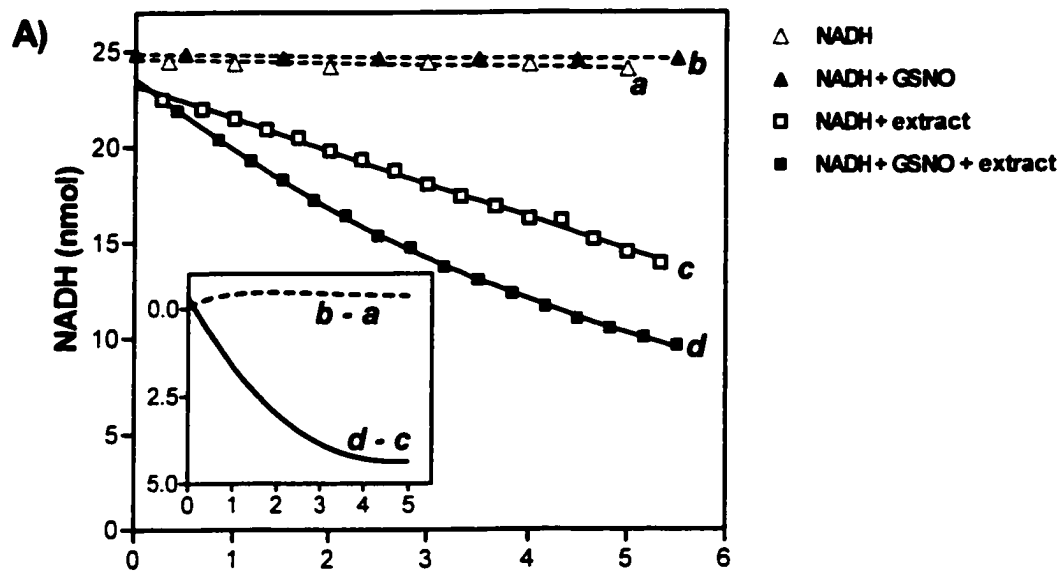
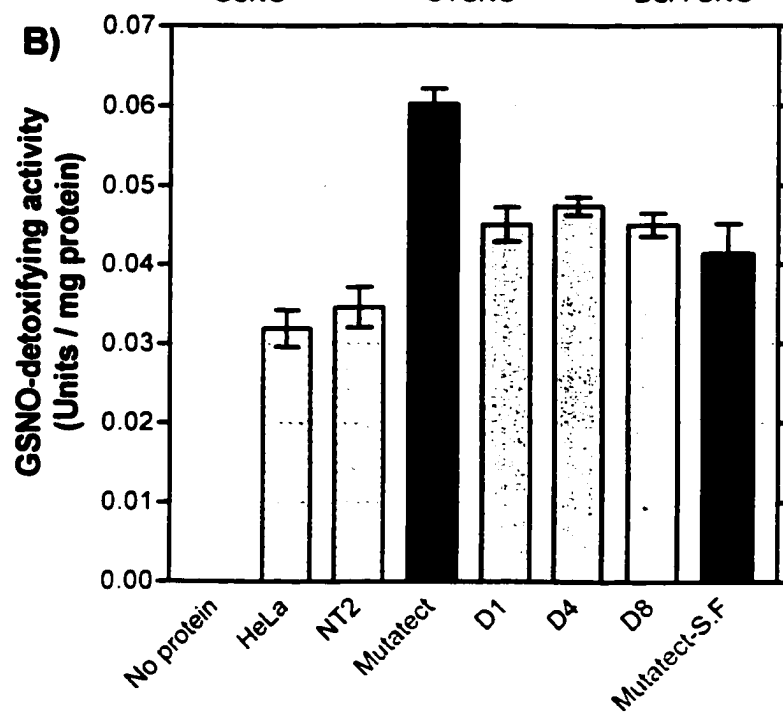
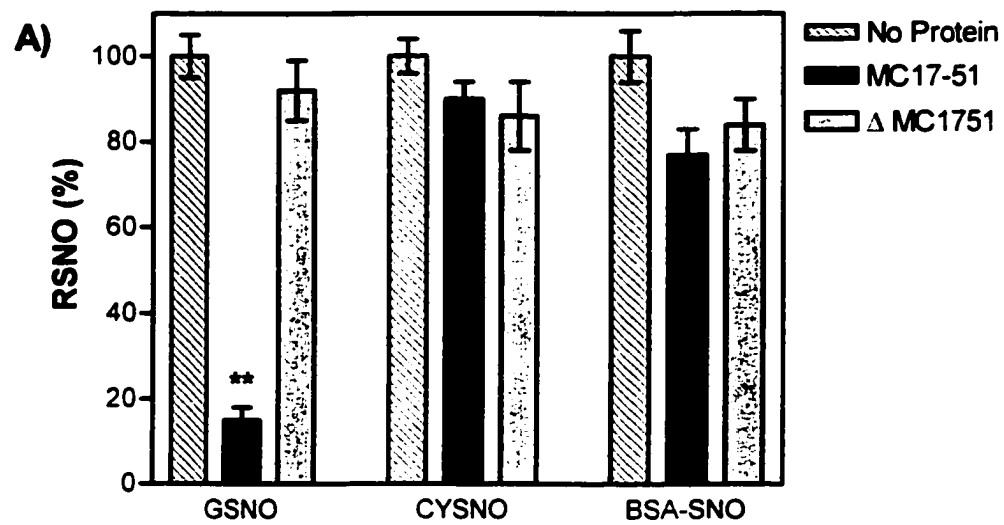


FIGURE 6.10. Specificity of GSNO-detoxifying activity and its levels in various cell lines and conditions.

A) Specificity of the detoxifying activity. GSNO, CYSNO or BSA-SNO were individually incubated with 50 mg of untreated Mutatect MC17-51 cell extract or heat inactivated extract (10 min at 75 °C) in the presence of NADH for 5 min and then assayed for the respective RSNO levels using the Saville-Greiss assay. **B)** Comparison of GSNO-detoxifying activity in HeLa, NT-2, Mutatect MC17-51, Mutatect tumors (D1, D4 and D8) and serum deprived Mutatect MC17-51 (Mutatect-S.F). One unit corresponds to 1 μ mol of GSNO detoxification per min. Each bar represents mean $\pm$ SEM of triplicate values. Other details in Materials and methods.



6.7. Discussion

The mechanism of NO_x-mediated mutagenicity is not well understood. Our lab was the first to demonstrate an involvement of intracellular GSH in NO_x-mediated mutagenicity using Mutatect cells *in vitro*. GSH was found to play a *pro-mutagenic* role in those experiments as it was required for the induction of mutations by NO_x-donating drugs. We therefore postulated that GSH is acting as a carrier of NO_x from cytoplasm to nucleus by forming intracellular GSNO. In this chapter, we initially set out to study the effect of altering GSH levels in Mutatect cells for their use in future animal experiments. Mutatect cell lines with high and low GSH levels were developed. However, we encountered a major “technical” problem, i.e., we could no longer demonstrate an induction of mutation by NO_x-donating drugs in Mutatect cells *in vitro*. In attempts to resolve the problem, we have obtained further insights into the possible mechanism of NO_x-mediated mutagenicity.

GSH levels were permanently manipulated in Mutatect cells by expressing sense or antisense mRNA of γ -GCS, the rate-limiting enzyme in GSH biosynthesis. Five stable MG clones with high GSH levels and one stable MAG-23 clone with low levels were identified. Cell growth and cell attachment appeared to be affected by GSH manipulation in these clones. In clone MAG-23, the lack of GSH was growth inhibitory. Administration of NAC, a cysteine pro-drug, rescued the slow growth for few days (<7); however, after a week, the cells started dying even in the continuous presence of NAC (not shown). GSH may be required here for maintaining normal cell growth, including maintenance of essential thiol status of proteins and storage of intracellular cysteine. The thiol status is required for most proteins to fold properly and be in an active state. It is possible that NAC, besides supplying intracellular cysteine, may transiently maintain the thiol status. However, NAC is also known to autooxidize and may liberate H₂O₂, causing cytotoxicity. Effect on cell attachment to culture plates was also observed by GSH manipulation. Clone MAG-23 did not adhere well to plates. On the contrary, MG clones adhered very strongly to plates. This suggests that GSH levels may play a role in cell adhesion in Mutatect cells. In fact, GSH levels have been shown to affect cell surface proteins (200). In addition, since GSH also maintains the essential thiol status of cell-surface proteins (71, 201), lack of GSH may lead to improper folding of these proteins, affecting adhesion. Finally, NO_x-mediated cytotoxicity was examined in MG and MAG cells. Consistent with the results by other workers, we found

that high levels of GSH were protective while low-levels sensitized cells towards NO_x-mediated cytotoxicity.

We were unable to induce NO_x-mediated mutagenicity in any of the Mutatect cell lines under conditions described previously by our laboratory. These include exposure to 1.0 mM SNP or 0.5 mM GTN for 24 h. However, the same conditions were capable of forming protein nitrotyrosine in the cells (see *section 5.6.3*). In addition, the *in vitro* conditions that led to the nitration of histones in the nucleus (i.e., 0.1 mM SNP exposure for ≥ 3 d) did not induce mutations (*section 5.6.3*). This appeared to suggest that the mechanism of protein nitrotyrosine formation may be different than the mechanism of mutagenesis; this is consistent with the apparent inability of dietary vitamin E to protect against nitrotyrosine formation *in vivo*, while able to protect against genetic instability (see *chapter 4*).

We considered possible reasons for the lack of mutagenicity by SNP or GTN *in vitro*:

- 1) Mutatect cells were not “mutatable”. This is not the case since Mutatect cells growing as subcutaneous tumors have a very high *hprt* mutation frequency, and we have shown that *hprt* mutants could readily be induced in Mutatect cell by X-irradiation (unpublished data). Thus, Mutatect cells could be mutated at the *hprt* locus.

- 2) Drugs were not releasing NO[•] or related species. We tested whether SNP and GTN were actually generating NO[•] or related species. Although neither of the drugs could generate NO[•], they both produced NO[•]-related species (NO_x). We also examined several RSNO (CYSNO, GSNO and SNAC) as NO_x-donors, although previous results from our laboratory have suggested that they were not as mutagenic as SNP or GTN. The RSNO were capable of releasing NO[•]. In addition, the rate of NO_x release was significantly higher than either SNP or GTN. CYSNO was found to be the most unstable under the conditions used hence had the highest rate of NO_x release. However, these drugs by themselves were not able to induce mutations. Thus, although the drugs were releasing NO[•] or related NO_x, they could not induce mutations at the *hprt* locus in the Mutatect cells *in vitro*.

- 3) Intracellular RSNO, and especially GSNO, was not formed. We examined whether intracellular RSNO was in fact formed after exposure to various NO_x-donating drugs. Only CYSNO, the highest NO_x-producer, was capable of inducing intracellular RSNO formation, albeit in small amounts. The majority of the RSNO formed was of high molecular mass, mainly corresponding to Pro-SNO, while GSNO did not appear to be

formed. Thus, the conditions that could not induce mutagenicity also were not forming detectable levels of intracellular RSNO, which we had postulated (especially GSNO) to be mutagenic. We then hypothesized that the conditions which would induce formation of high levels of intracellular RSNO, and especially GSNO, would also induce mutagenicity. After testing a number of different conditions, we found 2 types of conditions that could induce a high level of intracellular RSNO formation: *I*) overnight-pretreatment with 0.2-1 mM SNP plus 2 h-treatment with 1 mM RSNO (CYSNO, SNAC or GSNO); *II*) 5 min-treatment with 5 mM CYSNO. However, detectable level of intracellular GSNO was not produced by any of the conditions, and the majority of the RSNO formed was Pro-SNO. Condition *I* produced as high as 1.6 nmol Pro-SNO / mg protein, while condition *II* produced ~9 nmol / mg protein. Using condition *I*, the combination of SNP/CYSNO produced the higher Pro-SNO compared to either SNP/GSNO or SNP/SNAC combination. Only condition *I* was examined for the induction of mutagenicity at the *hprt* locus in Mutatect cells. A slight increase in *hprt* mutation frequency was seen by either SNP/GSNO or SNP/SNAC, which was weakly statistically significant. A *significant* increase in mutation frequency was observed by exposure to SNP/CYSNO, but this combination also caused a large amount of cytotoxicity (>95%). A more desirable condition would have been the one in which cytotoxicity by NO_x-donors was low (<50%) in order to study the effect of GSH depletion *in vitro*, since GSH depletion usually enhances the cytotoxicity. In condition *II*, the cell cytotoxicity was only 5%. The mutagenicity by this condition will be examined at a future time. Thus, taken all together, the combinations of NO_x-donating drugs that led to high intracellular RSNO formation also appear to induce high *hprt* mutation frequency in Mutatect cells.

4) the GSNO (if formed) was detoxified rapidly. We had hypothesized that GSNO is the carrier of mutagenic species in Mutatect cells. However, none of the NO_x-donating drugs or the combination of drugs used in this chapter was able to induce GSNO formation in the cells. Formaldehyde dehydrogenase (FDH) was recently shown to be able to specifically detoxify GSNO in a NADH-dependent manner in mammalian cells. This GSNO-specific detoxifying activity was also present in Mutatect cells. However, the activity was unusually high compared to i) other mammalian cells, ii) extracts of Mutatect tumors and iii) Mutatect cells growing in a culture medium free of fetal calf serum. This unusually high level of GSNO detoxification in Mutatect cells growing in the presence of fetal calf serum, likely due

to FDH over-expression, may be a possible reason for the lack of NO_x-mediated mutagenicity *in vitro* presently observed. Some factor(s) in fetal calf serum may be inducing the expression of FDH in Mutatect cells *in vitro* but not in other mammalian cells. In addition, the factor(s) may be absent in the tumor microenvironment, hence the FDH levels were low and the Mutatect cells were “mutable” *in vivo*. However, GSNO levels were not measured under these conditions. One reliable way to test these hypotheses and test whether GSNO is an intracellular mutagenic species is to down-regulate the GSNO-detoxifying activity in Mutatect cells (as done in the next chapter) and measure the NO_x-mediated mutagenicity.

6.8. Significance

Several significant findings were observed towards understanding the mechanism of mutagenicity by NO'-derived factors. Using the conditions described previously in our laboratory, we were unable to induce mutations in Mutatect cells by NO_x-donating drugs. However, these conditions also did not lead to any intracellular Pro-SNO or GSNO formation. This is consistent with our original hypothesis that GSNO acts as a carrier of intracellular NO_x. Preliminary results show that the formation of intracellular Pro-SNO may be associated with the induction of mutations in the tumor cells. As would be seen in *chapter 7*, the levels of Pro-SNO and GSNO may be regulated by a same metabolic cycle, involving FDH. Thus, the association between Pro-SNO and mutagenicity might also implicate an association between GSNO and mutagenicity. However, due to rapid detoxification of GSNO by unusually high levels of FDH in Mutatect cells, detection of GSNO may be below the sensitivity of the assay. Furthermore, the possible involvement of Pro-SNO in the process of mutagenicity can not be ruled out. For example, *S*-nitrosation of some DNA-repair enzymes has been shown to lead to their inhibition (202), which may further lead to un-repaired DNA damage and consequently induction of mutations. This hypothesis needs to be further examined.

The identification of the 2 methods for *S*-nitrosation of proteins also has significance. It allowed us to better understand the mechanism of Pro-SNO formation, which likely is different than that of protein nitrotyrosine formation. In addition, these methods were significantly useful in understanding the role of FDH in protein *S*-nitrosation (next chapter).

Furthermore, since these methods could induce *hprt* mutagenicity, they should be useful in the future in studying the effects of BSO on mutagenicity.

Two types of cell lines (MAG and MG) with altered levels of GSH were developed, which are likely to have future relevance. They indicated that GSH may play a role in cell adherence and confirmed the requirement of GSH in protection against NO_x-mediated cytotoxicity. They should be useful for examining the role of GSH levels in NO_x-mediated mutagenicity, as well as S-nitrosation, in cultured Mutatect cells using the 2 new methods mentioned above. These cells should also be useful at a future time for examining the role of GSH levels in mutagenicity *in vivo* in animal experiments.

6.9. Conclusions

Manipulation of GSH levels in Mutatect cells showed that a continuously low level of GSH is toxic to cells and that GSH may be required for the adherence of Mutatect cells to culture plates. In addition, we confirmed that high levels of GSH are protective and low levels are harmful towards NO_x-mediated cytotoxicity in Mutatect cells. We identified 2 conditions that could induce a high level of intracellular RSNO formation in Mutatect cells: *I*) overnight-pretreatment with 0.2-1 mM SNP plus 2 h-treatment with 1 mM RSNO (CYSNO, SNAC or GSNO); *II*) 5 min-treatment with 5 mM CYSNO. These also appear to induce *hprt* mutation frequency. Finally, Mutatect cells growing *in vitro* in complete medium were found to have unusually high levels of FDH.

Chapter 7. Formaldehyde dehydrogenase plays a regulatory role in NO_x-mediated S-nitrosation of proteins in Mutatect and other mammalian cells

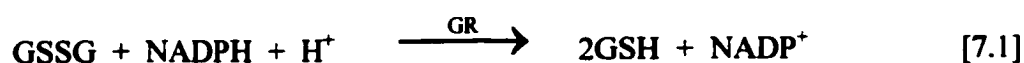
7.1. Chapter Summary

Reactive nitrogen oxide species (NO_x)-dependent protein *S*-nitrosation is a post-translational modification that has been shown to affect the activity of many important regulatory proteins. In *chapter 6*, two methods of inducing high level of cellular protein *S*-nitrosation by NO_x -donating drugs were developed to understand the role of thiols in the process of NO_x -mediated mutagenicity. While understanding this process, a possible cellular mechanism for the regulation of protein *S*-nitrosation was identified and is reported in this chapter. Initially, role of glutathione in the process of protein *S*-nitrosation in Mutatect cells was examined. Depletion of glutathione pools was found to dramatically enhance the formation of protein *S*-nitrosothiols, suggesting that glutathione protects proteins from being *S*-nitrosated likely by forming *S*-nitrosoglutathione (GSNO). Similar results were observed in HeLa cells. Recently, formaldehyde dehydrogenase (FDH) was shown to detoxify cellular GSNO. Therefore, role of FDH in protein *S*-nitrosation was examined. Mutatect cells contain 2-fold higher FDH activity than HeLa cells. The levels of both protein *S*-nitrosothiol and GSNO after exposure to NO_x -donors was significantly higher in HeLa cells than in Mutatect cells and was inversely related to their levels of GSNO-detoxifying activity. To further examine this, we genetically down regulated FDH levels in Mutatect cells by stably expressing antisense RNA or short-interfering RNA. Several Mutatect cell lines with low levels of FDH, one even lower than HeLa cells, were isolated. Levels of both protein *S*-nitrosothiol and *S*-nitrosoglutathione after exposure to NO_x -donors was significantly enhanced after down-regulation of FDH. A strong inverse correlation between the cellular total *S*-nitrosothiols and FDH activity was seen ($P < 0.006$, $r = 0.76$, $n = 11$). Reduced and oxidized forms of glutathione were also monitored, and the involvement of glutathione reductase in protein *S*-nitrosation was implicated. In addition, inhibition of glutathione reductase function using dehydroepiandrosterone was also attempted. The results implicate that FDH-dependent detoxification of *S*-nitrosoglutathione plays a key role in protecting against NO_x -mediated protein *S*-nitrosation. A glutathione-dependent metabolic cycle involving enzymes FDH and glutathione reductase that is capable of preventing protein *S*-nitrosation is proposed.

7.2. Introduction

Free sulfhydryl-containing molecules (RSH) can readily react with various NO_x species to form S-nitrosothiol (RSNO) in a reaction termed S-nitrosation or S-nitrosylation (15, 19, 20). In proteins, sulfhydryl groups are now considered as a major intracellular target of NO_x to produce protein RSNO (Pro-SNO). Pro-SNO formation has been shown to occur *in vivo* for a number of proteins (20). This post-translational modification has been shown to affect the activity of many important regulatory proteins, such as p21^{ras}, N-methyl-D-aspartate receptor, caspases, c-Src and nuclear factor-κB (20, 21). This type of protein modification is increasingly becoming recognized as a ubiquitous regulatory reaction comparable to protein phosphorylation (20). Pro-SNO and other RSNOs are also capable of *generating* different forms of NO_x (including NO[•], NO⁺, NO⁻, NO₂[•] or NO₂⁺) or promoting formation of N₂O₃ or ONOO⁻ (11, 195, 196). In fact, RSNOs are extensively used as NO_x-donating compounds. This property of RSNO has led to the postulate by several workers (20, 197) and our laboratory (77)(chapter 6) that RSH function as transporters of NO_x species. The NO_x-carrying property of glutathione (GSH), cysteine, hemoglobin and albumin has been postulated since their corresponding RSNO (GSNO, CYSNO, S-nitroso-hemoglobin and S-nitroso-albumin) have been detected and quantified *in vivo* (20, 89, 197). In fact, these RSNO may be responsible for some of the well-documented physiological processes that had been previously attributed to NO[•] itself (19).

GSH is the major non-protein sulfhydryl-containing molecule in mammalian cells (71). It is present in virtually all animal cells, often in relatively high concentrations (0.5-10 mM). The majority (>70%) of glutathione exists in the thiol-reduced (GSH) form; other forms include disulfide oxidized (GSSG) and mixed disulfide (GSSR). GSH can be derived from GSSG in an NADPH-dependent reaction catalyzed by glutathione reductase (GR) (79):

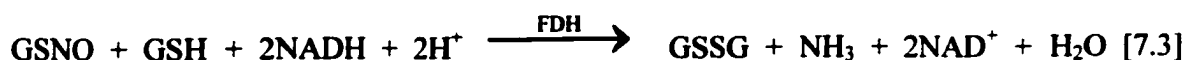


However, the *de novo* synthesis of GSH occurs by the sequential action of 2 enzymes: γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase (see Fig. 6.1). The reaction catalyzed by γ-GCS is the rate-limiting step in the biosynthesis of GSH and is a target of a specific and irreversible inhibitor L-buthionine-(S,R)-sulfoximine (BSO) (203).

GSH readily reacts with many NO_x species to form GSNO. *In vitro*, GSNO (and other low MW RSNO) are widely used to S-nitrosate proteins in a spontaneously reversible reaction termed *S-transnitrosation* (19, 197).



In vivo, the role of GSH in the process of protein S-nitrosation is not well-studied. It is believed that accessible –SH groups in proteins can be directly S-nitrosated by NO_x or indirectly *via* GSNO *in vivo*. Recently, however, rapid enzymatic detoxification of GSNO was described in living cells (90). Formaldehyde dehydrogenase (FDH; EC 1.1.1.1) was shown to catalyze this reaction in an NADH-dependent manner as follows:



FDH is the only known enzyme in mammalian cells that has been shown to possess denitrosating activity and hence may be important in regulating the level of intracellular NO_x. However, FDH is specific to GSNO and can not metabolize other RSNO, including Pro-SNO (90)(see also section 6.6.6). So far, no mechanism for the regulation of *in vivo* protein S-nitrosation has been reported. In chapter 6, we demonstrated two methods of inducing high level of protein S-nitrosation by NO_x-donating drugs in Mutatect cells. These included exposure to i) CYSNO-alone or ii) a combination of GSNO and SNP. Intracellular GSNO, which we postulate is a transporter of mutagenic species, did not appear to be formed under these conditions. We also found unusually high levels of FDH activity in Mutatect cells compared to HeLa and other cells, and postulated that this was responsible for the lack of GSNO detection and mutagenicity in the cells (chapter 6). While attempting to understand the mechanism of NO_x-mediated mutagenicity using the S-nitrosating conditions, we have found a possible mechanism for the regulation of protein S-nitrosation in mammalian cells. In this chapter, we describe a role of GSH in the process of protein S-nitrosation. In addition, we have genetically down-regulated the FDH levels in Mutatect cells. Using these cells, we show that FDH levels may play a key regulatory role in protein S-nitrosation. The effect of mutagenicity in these cell lines will be examined at a future time.

7.3. Hypotheses

The overall hypothesis was that GSH plays a key role in the process of protein S-nitrosation. Specifically, the formation of GSNO in cells might facilitate the formation of Pro-SNO. Based upon this, we also postulated that the levels of FDH – the GSNO-detoxifying enzyme – may act as a regulator of protein S-nitrosation.

7.4. Initial objectives and expected results

We initially set out to examine the following objectives:

1. *To examine the effect of GSH depletion (by BSO) in the process of protein S-nitrosation by NO_x-donating drugs in cultured cells.* It was expected that depletion of GSH would reduce protein S-nitrosation in cells since studies by others have suggested a role of GSNO in transnitrosation of proteins.
2. *To develop cell lines with reduced levels of FDH.*
3. *To examine the effect of reduced levels of FDH in the process of protein S-nitrosation by NO_x-donating drugs in cultured cells.* It was expected, based upon the expectation in objective 1, that the reduction in the GSNO-detoxifying activity (FDH levels) would reduce protein S-nitrosation.

7.5. Materials and methods

Chemicals. Sodium nitroprusside (SNP; reagent grade) was from Sigma-Aldrich Chemicals (St. Louis, MO), and was prepared immediately before use in Dulbecco's modified Eagle medium (DMEM) plus 10% fetal calf serum. Reduced GSH and cysteine hydrochloride (CYSH) were from Sigma-Aldrich Chemicals. L-Buthionine-(S,R)-sulfoximine (ICN Biomedicals Inc., Aurora, OH) was prepared immediately before use in PBS. GSNO and CYSNO or SNAC were made as described in *chapter 6*. Other reagents were from Sigma-Aldrich, unless otherwise stated.

Construction of FDH-expressing constructs. To make antisense constructs, an I.M.A.G.E. Consortium LLNL cDNA clone #2812535 was identified to contain mouse FDH sequences and obtained from a commercial source, as described in *chapter 1*. The clone was digested completely with Not I to isolate the insert (~1.4 kb) containing the full-length mouse FDH cDNA. A mammalian expression vector pCR3 (Invitrogen, Carlsbad, CA) was

also digested with Not I and ligated with the insert. A construct pCMVmFDH-Full-Anti (see *Appendix A1*) containing the full-length cDNA of FDH under the cytomegalovirus promoter in an antisense orientation was identified by restriction nuclease analyses. To create a 5'-end-only containing antisense construct, pCMVmFDH-Full-Anti was completely digested with Pst I and a ~5.1 kb fragment was isolated and ligated to itself. The resulting construct pCMVmFDH-5' end (not shown) contained the entire 71 bp of 5'-untranslated region of FDH plus 29 bp of coding-region in antisense direction under the cytomegalovirus in pCR3.

To make constructs capable of expressing short-interfering RNA (siRNA), the following approach was taken. Two constructs were made, one targeting the 5'-end of the FDH mRNA, the other targeting the mid-portion. The 5'-end construct targeted the sequence from -18 to +4 of the mRNA, including the AUG. To make this construct, two oligodeoxynucleotides were made one of them was 5'-phosphorylated: AGCTGATCCCGAACTAGCGGCCATGGATTAAT and PCCATGGCCGCTAGTTCGGGATC. These were annealed to give the following fragment:



This fragment was then ligated to itself. Ligation occurred only at where 5'-phosphorylation was present. The resulting fragment (containing AGCT overhangs) was ligated to Hind III-digested pCR3 to produce a construct that was predicted to be capable of expressing the following double-stranded RNA structure.



The construct targeting mid portion of the mRNA was made similarly, except that the following oligodeoxynucleotides were used:

AGCTGCCACACCGATGCCTATACCCATTAAT and
PGGGTATAGGCATCGGTGTGGC.

Conditions for cell culture and transfections. See chapter 1 for details.

Exposure of cells to NO_x donors. See chapter 6.

Low MW RSNO and Pro-SNO measurements. See chapter 6 for details.

Protein measurements. Fluorescamine was used to quantify protein (147).

GSNO-detoxifying activity. See chapter 6 for details. One enzymatic unit is defined as 1 μ mol of GSNO metabolized per min.

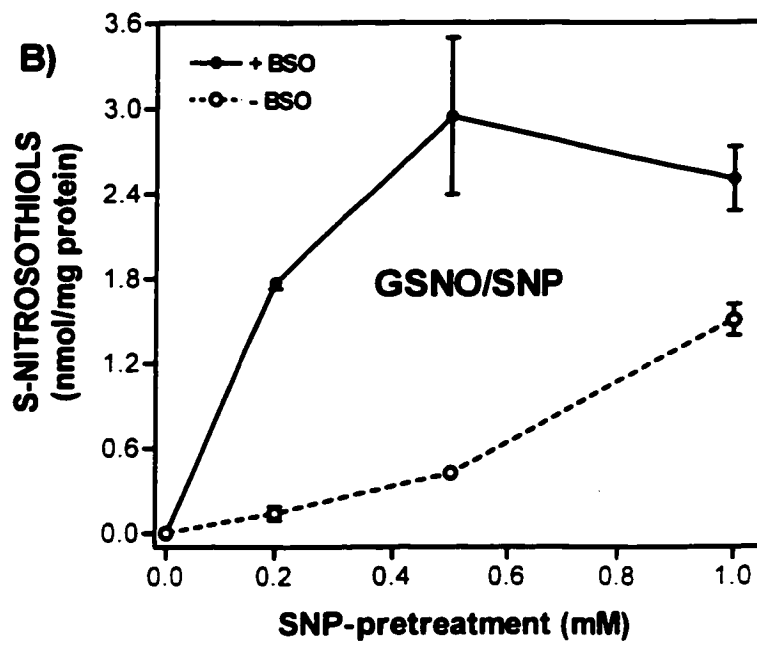
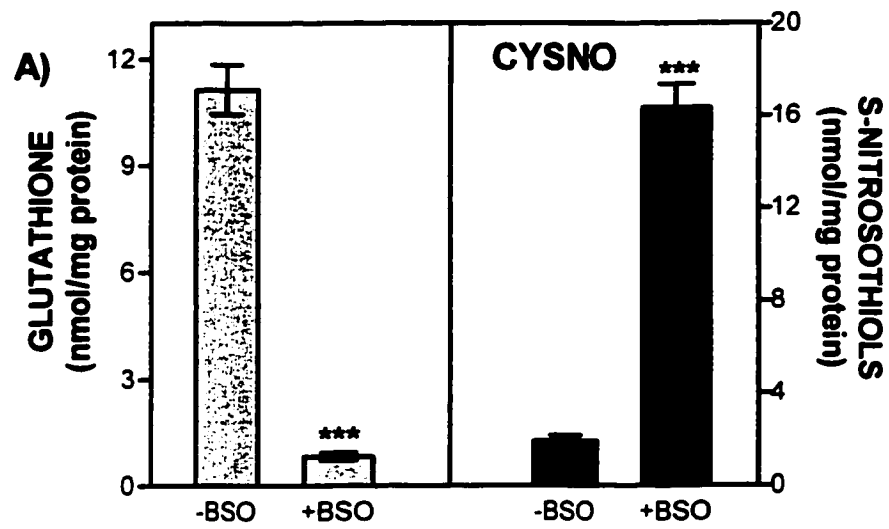
7.6. Results

7.6.1. Effect of GSH depletion on NO_x-dependent RSNO formation in Mutatect cells

We have earlier demonstrated that intracellular RSNO could be formed in cultured Mutatect cells after exposure to certain NO_x-donors (see section 6.6.4); these included either exposure to CYSNO-alone or to a combination of GSNO/SNP. To examine whether GSH plays a role in the process of *S*-nitrosation, cells were depleted of their GSH pools using BSO and analyzed for RSNO formation by the NO_x-donors. BSO is a specific and irreversible inhibitor of γ -GCS, the rate-limiting enzyme of the *de novo* biosynthesis of GSH. Exposure of BSO to Mutatect cells for 24 h led to ~95% reduction in GSH levels (Fig 7.1A). When these cells were exposed to CYSNO, a statistically significantly higher level of RSNO was detected in the extract of the cells; the level was 8 $\times$ higher than the level in cells not treated with BSO (Fig 7.1A). After fractionation of the extracts, it was found that ~95% of the total RSNOs were of high-MW, corresponding to Pro-SNO, while only ~5% were of low-MW (Table 7.1). The low-MW RSNO were likely CYSNO and not GSNO, since the concentration was unaffected in the presence or absence of BSO (Table 7.1). Similar results were observed in cells exposed to GSNO/SNP in the absence or presence of BSO (Fig 7.1B). However, *S*-nitrosation was much lower using GSNO/SNP than CYSNO-alone (*cf.* Fig 7.1A and B). In addition, low-MW RSNO was not detectable (data not shown). Thus, these results show that cellular pools of GSH dramatically *protect* proteins from being *S*-nitrosated by NO_x-donating compounds.

FIGURE 7.1. Effect of GSH depletion by BSO on NO_x-dependent RSNO formation in Mutatect MC17-51 cells.

Where indicated, cells were treated with 0.1 mM BSO for 48 h prior to treatment with NO_x-donors. **A)** GSH levels (*left panel*) and CYSNO-dependent RSNO formation (*right panel*) in the absence or presence of BSO. **B)** GSNO/SNP-dependent RSNO formation in the absence or presence of BSO. Each point represents mean $\pm$ SEM of triplicate values. Other details are in Materials and methods.



7.6.2. Mutatect vs. HeLa cells: NO_x-mediated RSNO formation and GSH levels

Mutatect cells contain about 2-fold higher GSNO-detoxifying activity than HeLa cells (section 6.6.6). To examine whether intracellular formation of RSNO by NO_x-donors would be different between the two cell-types, both Mutatect and HeLa cells were exposed to either CYSNO or GSNO/SNP and their extracts analyzed for total RSNO. After exposure to either type of NO_x-donor, the RSNO level was about 3-to-5-fold higher in HeLa extracts than in Mutatect extracts (Fig. 7.2A). To examine the type of the thiols that were S-nitrosated, the extracts were fractionated into low-MW and high-MW by acid precipitation and analyzed. Both low- and high-MW RSNO were significantly higher in HeLa cells than in Mutatect cells after CYSNO-exposure (Fig. 7.2B): low-MW RSNO was 15-fold higher and Pro-SNO was 4-fold higher. The low MW RSNO were likely GSNO, since depletion of GSH by BSO prior to CYSNO exposure also led to depletion of low MW RSNO (Table 7.1). Thus, these results show that the level of both Pro-SNO and low-MW RSNO formation after exposure to NO_x-donors is significantly higher in HeLa cells than in Mutatect cells, and is inversely related to their levels of GSNO-detoxifying activity.

Cellular GSH levels were found to be protective against NO_x-mediated RSNO formation (see above 7.6.1). To examine whether GSH levels in HeLa cells were lower than Mutatect cells and this was responsible for the high RSNO formation, GSH was measured in both HeLa and Mutatect cells using a fluorometric assay. As shown in Fig. 7.2C, the GSH level in HeLa was not lower than the level in Mutatect cells; in fact, the level was about 40% *higher*. Thus, the high level of RSNO formation in HeLa cells was independent of GSH levels and more likely a result of low level of GSNO-detoxifying activity.

7.6.3. Permanent down-regulation of formaldehyde dehydrogenase in Mutatect cells

Detoxification of GSNO was recently shown to be catalyzed by formaldehyde dehydrogenase (FDH) (90). Development of Mutatect cell lines with permanently-suppressed levels of FDH was accomplished by stably over-expressing either antisense mRNA or siRNA of FDH. Two antisense-expressing constructs were developed: one targeting the full-length of the mRNA and other targeting only the 5'-end of the mRNA. Two siRNA-expressing constructs were also developed: one targeting 21 bp of the 5'-end of mRNA (including the start codon) and the other targeting 20 bp within the coding region.

FIGURE 7.2. Mutatect vs. HeLa cells: NO_x-mediated RSNO formation and GSH levels.

A) RSNO induced by either CYSNO (left axis, filled bars) or GSNO/SNP (right axis, hatched bars) in Mutatect, HeLa or BSO-treated HeLa. **B)** Levels of low MW RSNO and Pro-SNO (high MW) induced by CYSNO in Mutatect and HeLa cells. **C)** GSH levels in Mutatect and HeLa cells. Each point represents mean $\pm$ SEM of triplicate values. Other details are in Materials and methods.

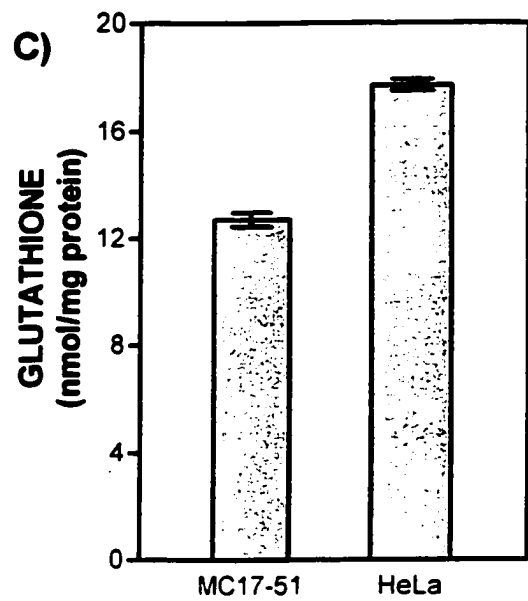
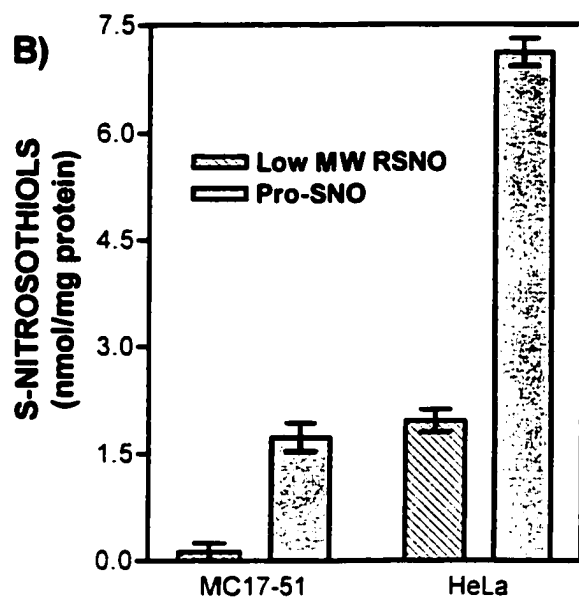
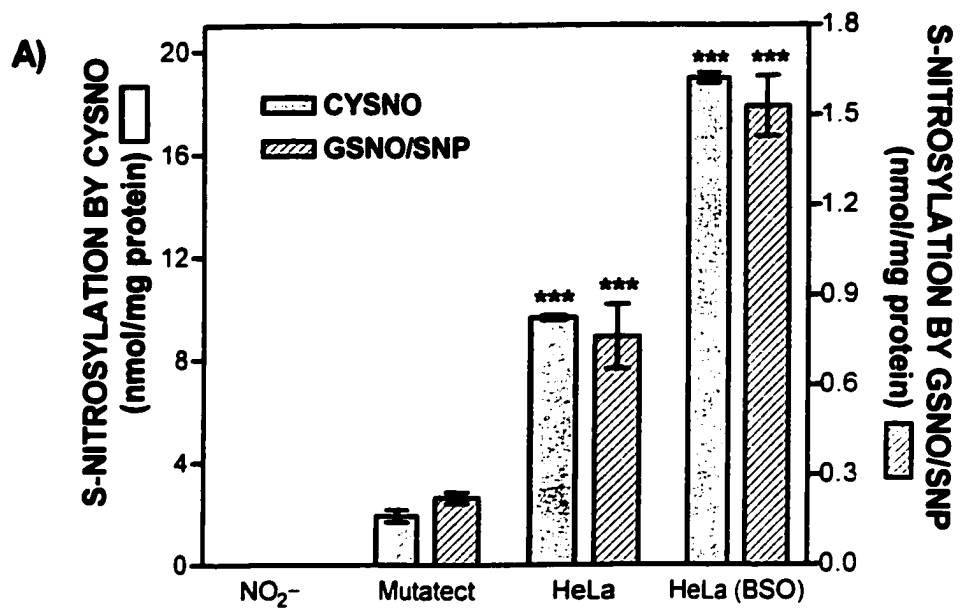


TABLE 7.1: Intracellular levels of low and high MW RSNO formed after exposure to CYSNO in the absence or presence of BSO in cells

Cells	RSNO concentration (nmol/mg protein)			
	– BSO		+ BSO	
	Low MW	High MW	Low MW	High MW
MC17-51	0.22	1.8	0.21	16
HeLa	1.9	6.2	0.51	18
MiF-6	2.0	7.9	0.31	17

Each of these constructs was transfected into Mutatect MC17-51 cells and stable clones were isolated on the basis of a linked neomycin-resistant gene. More than 30 antisense-expressing clones (named MAF clones) and 12 siRNA-expressing clones (named MiF clones) were isolated. Extracts from the clones were analyzed for the level of GSNO-detoxifying activity and compared with the levels in M-7 clone (a vector-alone transfected clone - see *section 6.1.1*) and parental MC17-51 cells (Fig. 7.3A and B). At least one of the 5'-end-antisense-expressing clone, MAF-1, was identified to have 34% lower GSNO-detoxifying activity than the controls (Fig. 7.3A). None of the full-length antisense-expressing clones had altered levels of the activity. On the other hand, at least 3 of the 5'-end-targetted siRNA-expressing clones, MiF-1, 6 and 8, were identified to have 19, 60 and 32% lower activity than the controls, respectively (Fig. 7.3B). None of the coding-region-targetted siRNS-expressing clones had altered levels of the activity. Thus, these results show that several Mutatect cell-lines with permanently low-levels of GSNO-detoxifying activity could be constructed by stably expressing antisense-mRNA or siRNA of FDH.

7.6.4. NO_x -mediated RSNO formation and GSH levels in Mutatect MAF and MiF clones

The effect of lowering the level of FDH on the level of NO_x -mediated RSNO formation in Mutatect cells was next examined. Mutatect MAF-1, MiF-1, MiF-6 and MiF-8 cells were identified to have 19-60% lower FDH activity than controls M-7 and MC-1751 cells (see above). These cells were exposed to an NO_x -donor, CYSNO, and their extracts analyzed for total RSNO. A significantly higher level of RSNO was detected in extracts of MAF and MiF cells, which was 3-to5-fold higher than the controls (Fig. 7.4A). In fact, in the case of MiF-6 cells, which had 60% lower FDH activity than the Mutatect controls, the level of RSNO formed was even higher than the level detected in HeLa cell extracts. RSNO levels were also measured in fractionated extracts of MC17-51, M-7, MAF-1, MiF-6 and HeLa cells after exposure to CYSNO (Fig 7.4B). The enhancement in RSNO levels in MAF-1 and MiF-6 cells compared to controls was found to be due to enhancement of both Pro-SNO as well as low-MW RSNO. The low MW RSNO were likely GSNO, since depletion of GSH by BSO prior to CYSNO exposure also led to depletion of low MW RSNO (Table 7.1). Thus, these results show that the level of NO_x -mediated formation of both Pro-

FIGURE 7.3. Permanent down-regulation of formaldehyde dehydrogenase (FDH) in Mutatect cells.

A) Mutatect MAF clones. MC17-51 cells were stably transfected with an antisense of FDH, targetting 5'-end of the mRNA, and neomycin-resistant clones were isolated. NADH-dependnet GSNO-detoxifying activity was analyzed in these clones to measure the FDH levels. **B) Mutatect MiF clones.** MC17-51 cells were stably transfected with an siRNA-expressing construct of FDH, also targetting 5'-end of the mRNA, and neomycin-resistant clones were isolated. NADH-dependnet GSNO-detoxifying activity was also analyzed in these clones to measure the FDH levels. Each point represents mean $\pm$ SEM of triplicate values. Other details are in Materials and methods.

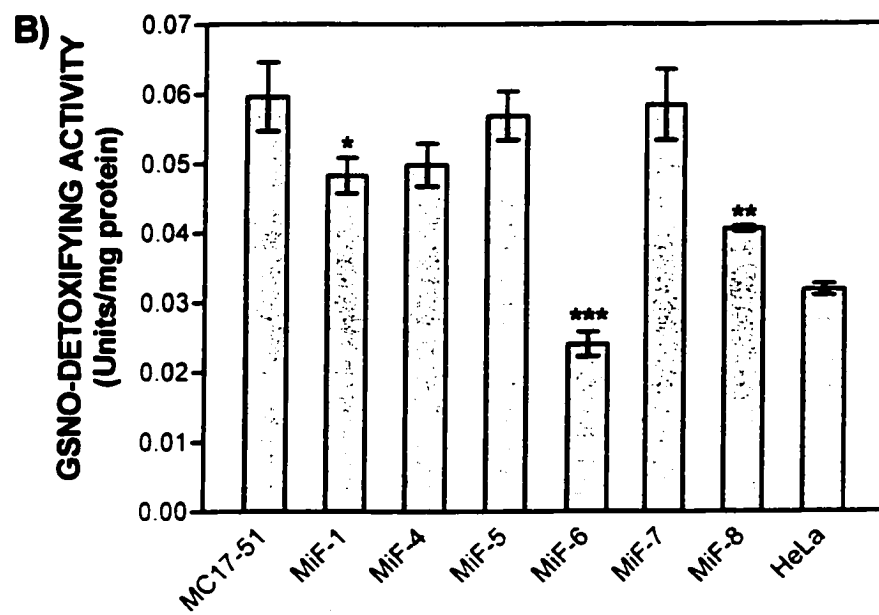
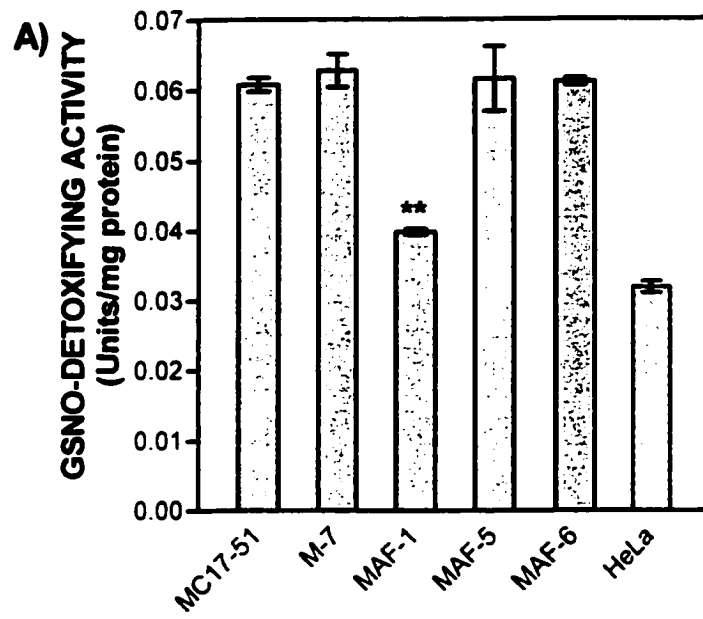
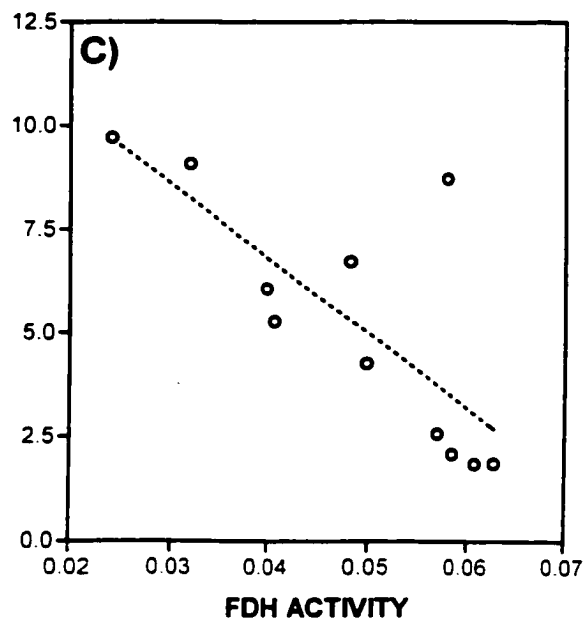
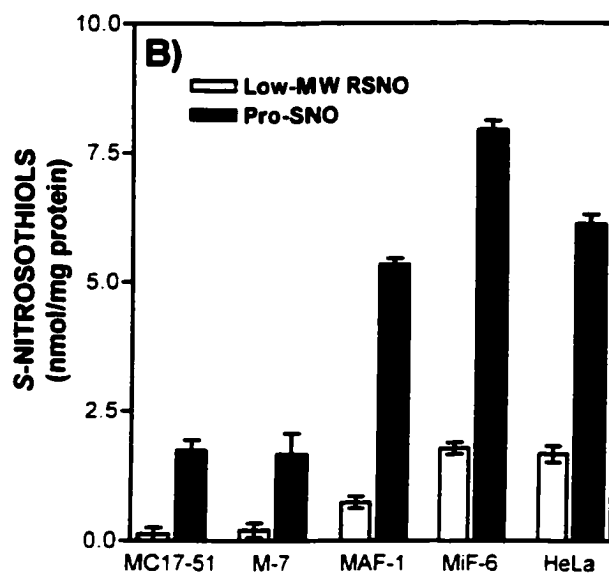
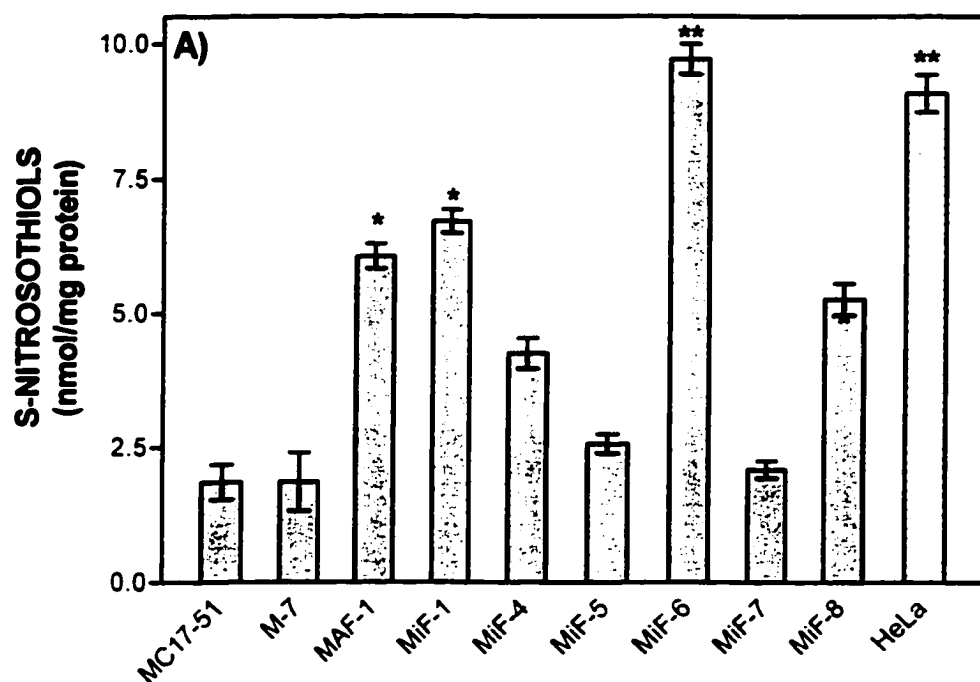


FIGURE 7.4. Effect of altered FDH levels on RSNO formation.

A) Total RSNO formed in various MAF and MiF clones after exposure to CYSNO. B) Levels of low MW RSNO and Pro-SNO (high MW) formed in MAF and MiF clones after CYSNO exposure. C) Correlation between total RSNO levels and FDH in various MAF and MiF clones, as well as in control M-7 and MC17-51 cells. Spearman Rank: $P < 0.006$, $r = 0.76$, $n = 11$. Each point represents mean $\pm$ SEM of triplicate values. Other details are in Materials and methods.



SNO and low-MW RSNO is significantly enhanced in Mutatect cells with permanently low-levels of FDH.

The results were also analyzed to determine whether a correlation exists between the intracellular level of total RSNO and the level of FDH activity. A strong inverse correlation between the two was seen ($P < 0.006$, $r = 0.76$, $n = 11$) (Fig. 7.4C). This suggests that FDH likely plays a key protective role against NO_x -mediated S-nitrosation of protein and GSH in cells.

7.6.5. GSH and GSSG levels in Mutatect cells after exposure to NO_x -drugs

We examined the possibility that low GSH levels in MAF and MiF clones was responsible for the high level of RSNO formation seen in 7.5.4. GSH level was measured in these cells and compared with control Mutatect cells. As shown in Fig. 7.5A, GSH levels in all the Mutatect cell lines were very similar. Thus, the high level of GSNO and Pro-SNO formation in MAF and MiF cells was independent of GSH levels and was more likely a result of low levels FDH.

GSSG is one of the products of the FDH-mediated detoxification of GSNO (equation 7.2). We next examined the effect of FDH down-regulation on other glutathione-containing low MW molecules in the absence or presence of NO_x -donating drugs. Levels of GSH, GSNO and GSSG were measured in M-7 and MiF-6 cells in the absence or presence of CYSNO (Fig. 7.5B). In the absence of the drug, both M-7 and MiF-6 cells had similar levels of GSH/GSSG and no GSNO. In the presence of the drug, both cells showed a decrease in GSH level; however, the decrease was much higher in MiF-6 than M-7 cells (Table 7.2). An increase in GSSG was seen in M-7 but no in change in MiF-6 cells. Consistent with the results shown above, GSNO was only detected in MiF-6 cells. In total, a decrease in the low MW glutathione-containing molecules was seen, which was likely due to the formation of mixed disulfides with protein (Pro-SSG). Thus, FDH levels appear to affect the ratios of GSH and GSSG in Mutatect cells.

7.6.6. Effect of DHEA on NO_x -mediated RSNO formation in Mutatect cells

Glutathione reductase (GR) is known to reduce GSSG into GSH in the presence of NADPH (see equation 7.1). Since the experiments above show that the ratio of GSH/GSSG

FIGURE 7.5. Glutathione status in Mutatect clones.

A) GSH levels in MC17-51, M-7, MAF-1 and MiF-6 clones. The bars represents mean $\pm$ SEM. **B)** Changes in GSH, GSSG and GSNO levels in clones M-7 and MiF-6 in the absence or presence of CYSNO. See Materials and methods for details.

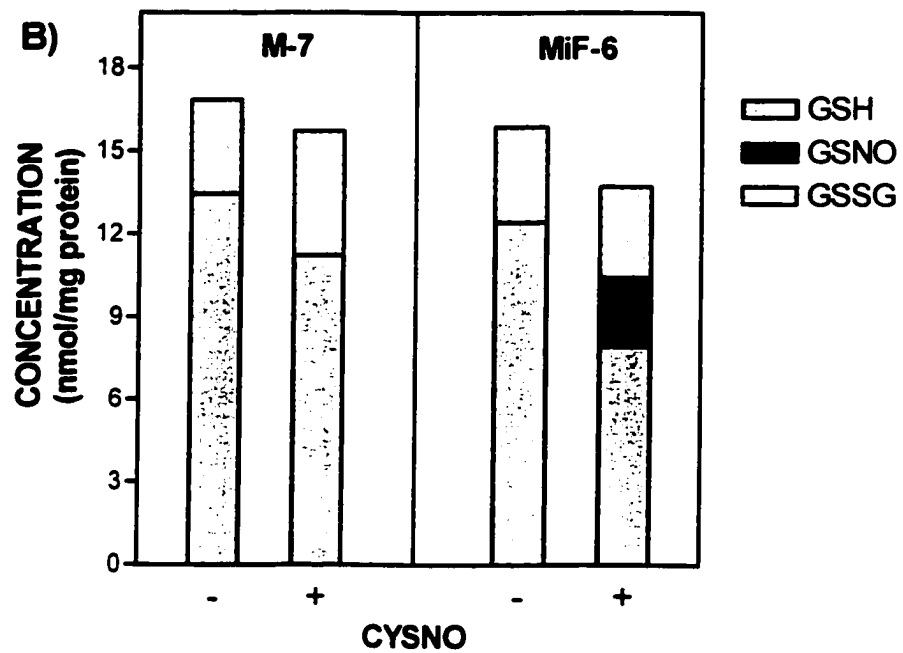
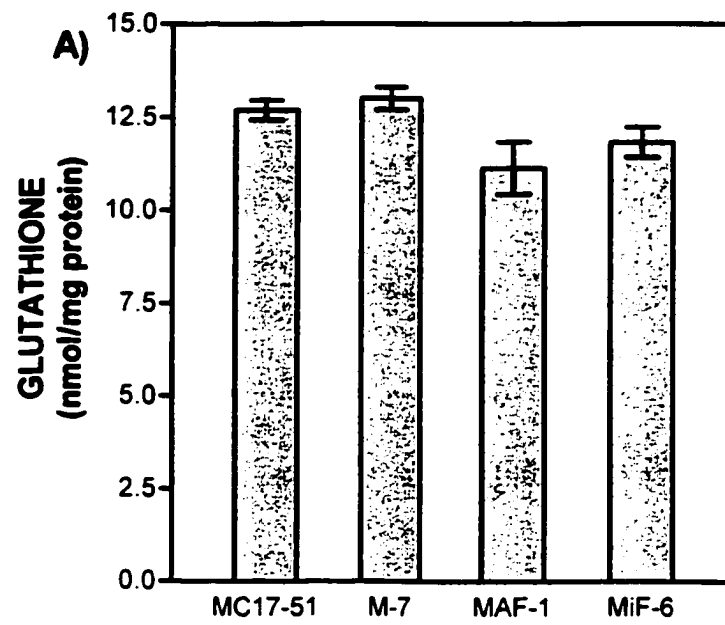


TABLE 7.2: Changes in GSH, GSNO, GSSG and Pro-SNO levels after exposure to CYSNO in Mutatect M-7 and MiF-6 cells

Cells	Change in concentration (nmol/mg protein)				
	Low MW ^(a)				High MW ^(b)
	GSH	GSNO	GSSG	Total	Pro-SNO
M-7	– 2.3	+ 0.0	+ 1.1	– 1.0	+ 1.7
MiF-6	– 4.5	+ 2.5	+ 0.0	– 2.0	+ 8.0

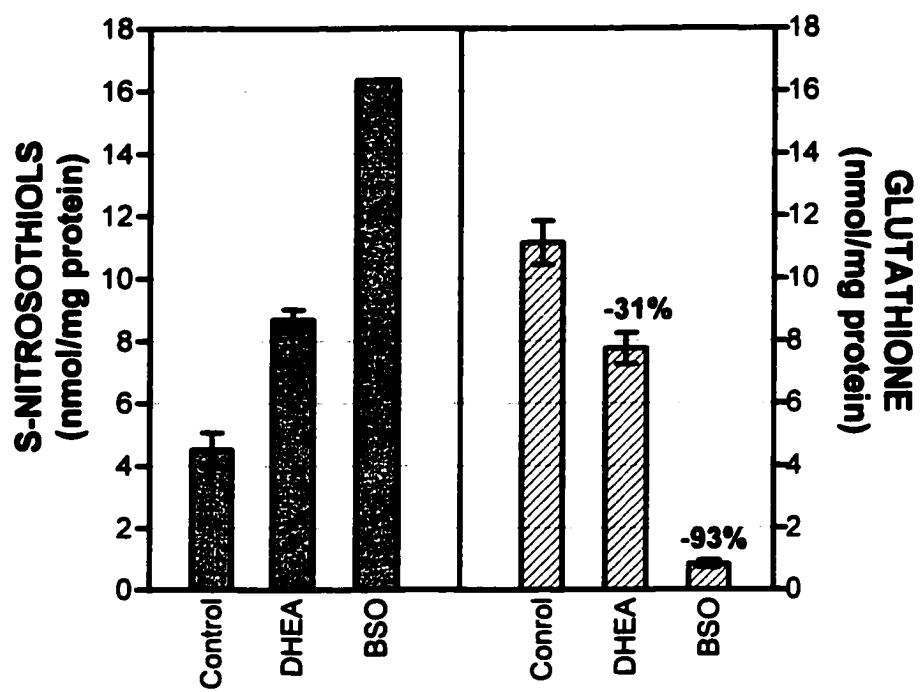
^(a) Data is calculated from Fig. 7.5.

^(b) Data obtained from Fig 7.4B.

is affected after exposure to NO_x-donating drugs, we examined the role of GR in protein S-nitrosation. A specific inhibitor is not available. However, dehydroepiandrosterone (DHEA), an inhibitor of glucose-6-phosphate dehydrogenase, has been used previously to deplete NADPH levels (204) and hence lower GR function in cells. We therefore exposed cells to DHEA and examined for protein S-nitrosation by CYSNO (Fig. 7.6). An almost 2-fold increase in Pro-SNO was seen in DHEA treated cells. To examine whether GSH level was affected in the cells after DHEA treatment, the levels of GSH were also measured. A 31% decrease in GSH was observed (Fig. 7.6). This was consistent with other reports that NADPH-depletion by DHEA lowers GR activity and hence decreases the salvage-synthesis of GSH. Thus, the increase in protein S-nitrosation seen after DHEA was likely due to a decrease in GSH levels.

FIGURE 7.6. Effect of DHEA pre-treatment on RSNO formation in Mutatect MC17-51 cells.

Left panel, Intracellular RSNO induced by CYSNO in the absence (control) or presence of DHEA. Cells were pretreated with 0.1 mM DHEA for 4 h. The effect of BSO on RSNO formation, originally shown in Fig. 7.1, is also included as a control. *Right panel*, effect of DHEA (and BSO) on the level of GSH. Each point represents mean $\pm$ SEM of triplicate values. Other details are in Materials and methods.



7.7. Discussion

A growing body of evidence indicates the relevance of *S*-nitrosation of peptides (such as GSH) and proteins in a variety of biological systems that either produce or were exposed to NO[•] or related NO_x species (15, 19-21, 197). The function of a number of proteins and multiple enzymes can be altered by *S*-nitrosation. Recent studies demonstrated the inhibition or modulation of important signalling pathways such as apoptosis through the *S*-nitrosation of proteins (20). The role of abundantly present GSH in protein *S*-nitrosation *in vivo* has not been previously demonstrated, although *S*-nitrosated GSH (i.e., GSNO) is able to *S*-transnitrosate protein *in vitro*. In addition, the mechanism of regulation of protein *S*-nitrosation is not known. In this chapter, we have shown that GSH does play a key role in protein *S*-nitrosation. We also have identified a possible mechanism of regulation of protein *S*-nitrosation *in vivo*.

GSH is present at high concentrations in virtually all animal cells. In Mutatect cells, the GSH level is 12 nmol/mg protein (~0.6 mM), which corresponds to 1 : 14 molar ratio between GSH and total cysteine residues in protein. However, since about half of the cysteine in protein are either disulfide bonded or involved in metal-binding (205), the ratio between GSH and *free* cysteine in proteins is more likely to be 1 : 7. Furthermore, due to the hydrophobic nature of cysteine residues, the majority of the free cysteine may not be accessible. Thus, the free accessible sulfhydryl groups in GSH might even out-number those in proteins. Due to its abundance and its ability to *S*-transnitrosate protein *in vitro*, we initially postulated that GSH may facilitate the NO_x-mediated protein *S*-nitrosation *in vivo*. However, the opposite, but not surprising, effect was found. GSH was found to protect proteins from *S*-nitrosation by NO_x-donating drugs in Mutatect and HeLa cells. This is consistent with the antioxidant role of GSH (71), as it is acting to protect cells from NO_x-mediated damage.

GSH readily reacts with NO_x species to form GSNO. However, GSNO formation was not detectable in Mutatect cells exposed to NO_x-donors (*chapter 6*). The fact that GSH levels protect against NO_x-mediated protein *S*-nitrosation suggests an interaction between GSH and NO_x that likely led to the formation of GSNO, albeit for a small period of time.

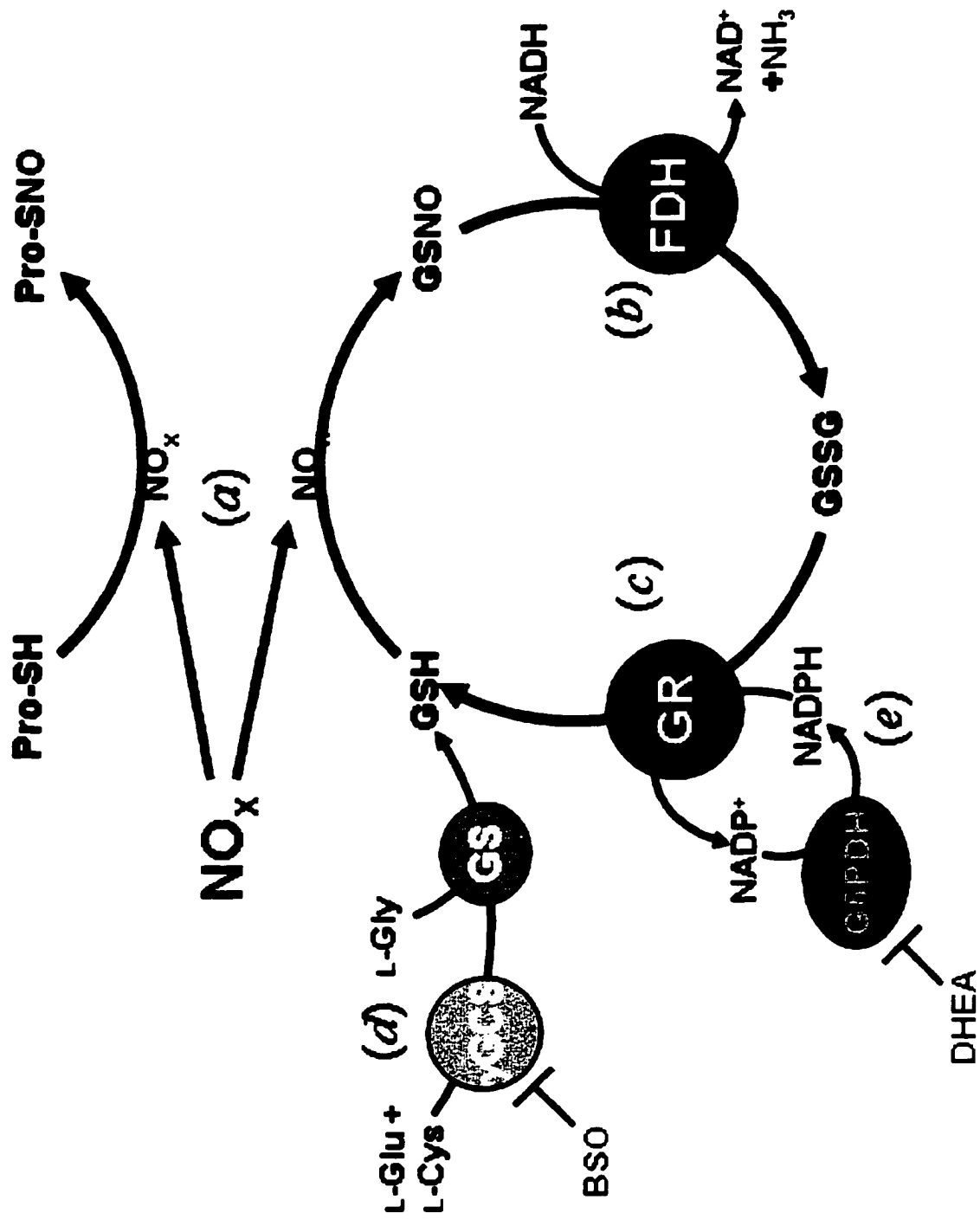
This is consistent with a decrease in GSH seen after exposure to NO_x-donors (Table 7.2). However, due to the presence of FDH, GSNO is believed to be rapidly detoxified.

Involvement of FDH – the GSNO-detoxifying enzyme – in protein S-nitrosation was demonstrated in cell lines with lower levels of FDH than Mutatect cells. HeLa cells were shown in *section 6.6.6* to contain 50% lower GSNO-detoxifying activity than Mutatect cells. A 4-fold higher level of Pro-SNO was seen in HeLa cells than Mutatect cells after exposure to NO_x-donating drugs. In addition, a large amount of GSNO was also detectable in HeLa cells, whereas none appeared to be present in Mutatect cells. This led us to believe that the high level of FDH in Mutatect cells may be responsible for the lack of GSNO detection. In addition, since GSH levels were protective, likely forming GSNO for a short period of time, we postulated that FDH levels were also important in protein S-nitrosation. To prove this, we set out to genetically manipulate FDH levels in Mutatect cells using antisense and RNA interference strategies. Five cell lines with permanently low levels of FDH were successfully developed. One of the clones, MiF-6, was identified to have ~60% lower FDH level than the parental Mutatect cells. This level was even lower than the HeLa cells. This clone behaved very similar to HeLa cells, with respect to intracellular RSNO formation after exposure to NO_x-donors. A large amount of both Pro-SNO and GSNO was detectable in this clone. This confirmed that a high level of FDH in parental Mutatect cells was responsible for the lack of GSNO detection. In addition, it confirmed the role of FDH in protein S-nitrosation.

To best understand our results, we propose a metabolic cycle involved in the regulation of protein S-nitrosation (Fig. 7.7). According to this model, NO_x can directly react with sulfhydryl groups of either GSH or Pro-SH in cells to produce the respective RSNO (GSNO or Pro-SNO) (scheme *a*). Since GSH is present at high concentration and more accessible, more GSNO is likely to be made. Depletion of GSH-containing molecules by BSO (scheme *d*) enhanced Pro-SNO levels. We interpret this to mean that GSH is competing with Pro-SH for NO_x. If the level of FDH is high, GSNO is readily detoxified into GSSG and ammonia (scheme *b*). This is consistent with the high level of GSSG seen in Mutatect M-7 cells, which have a high level of FDH. GSSG is known to be reduced by GR in an NADPH-dependent reaction (scheme *c*). Regenerated GSH may again react with NO_x

FIGURE 7.7. A proposed metabolic cycle involved in the regulation of S-nitrosation of protein and glutathione.

See discussion for explanation. *Abbreviations:* NO_x, reactive nitrogen oxide species; Pro-SH, accessible sulfhydryl groups in protein; Pro-SNO, S-nitrosated proteins; GSH, reduced glutathione; GSNO, S-nitroso-glutathione; FDH, formaldehyde dehydrogenase; GSSG, glutathione disulfide; GR, glutathione reductase; G6PDH, glucose-6-phosphate dehydrogenase; DHEA, dehydroepiandrosterone; BSO, buthionine sulfoximine; γ -GCS, γ -glutamylcysteine synthetase; GS, glutathione synthase.



and continue the metabolic cycle. In MiF-6 cells, because of the low level of FDH, the cycle may not be as active. Thus, a large amount of GSNO is present and GSH is not regenerated as much, consistent with the low levels of GSH seen after NO_x-exposure in these cells (Table 7.2). However, accumulation of GSSG is not seen in MiF-6, whereas it is seen in M-7 cells (Table 7.2). To explain this we postulate that, in M-7 cells, the rate-limiting step in the cycle is the reaction catalyzed by GR. In MiF-6 cells, due to a low level of FDH, the rate-limiting step may be the reaction catalyzed by FDH. Finally, our attempt to inhibit the GR function using DHEA-mediated depletion of NADPH (e) was not very conclusive. This was due to an inhibition in the salvage pathway leading to a decrease in GSH levels (although it further confirmed the role of GSH in protein *S*-nitrosation). However, a more informative experiment would be to compare the ratio of GSH/GSSG in M-7 and MiF-6 cells after treatment with NO_x-drugs and DHEA.

7.8. Significance

Protein *S*-nitrosation is a post-translational modification that has been shown to affect the activity of many important regulatory proteins and is increasingly becoming recognized as a ubiquitous regulatory reaction comparable to protein phosphorylation. Regulation of protein *S*-nitrosation is, therefore, expected to have biological relevance. The mechanism of this regulation has not been previously demonstrated. FDH was recently described to contain denitrosylating activity specific to GSNO and was suggested to be an important mechanism in protection against NO_x-mediated effects. We here show specifically that this reaction protects against NO_x-mediated protein *S*-nitrosation. A GSH-dependent metabolic cycle involving enzymes FDH and GR that is capable of preventing protein *S*-nitrosation was proposed.

Distribution and level of FDH are known to vary among different tissues (86, 206, 207). Significance of this variability is usually recognized in terms of the “classical” property of FDH, namely its ability to detoxify formaldehyde. The identification of the novel functions of FDH including detoxification of GSNO and regulation of protein *S*-nitrosation suggests a possible biological significance of NO_x-dependent effects in these tissues. Thus, distribution and level of Pro-SNO is also expected to be variable in these tissues, assuming that they are exposed to NO[•].

In *chapter 6*, we had hypothesized that GSNO is the carrier of mutagenic species in Mutatect cells. However, none of the NO_x-donating drugs or the combination of drugs used was able to induce GSNO formation in the cells. This was attributed to the unusually high levels of FDH in Mutatect cells. In the present chapter, several Mutatect cell lines with low levels of FDH were developed. High levels of Pro-SNO *and* GSNO could be formed in these cells after exposure to NO_x-donating drugs. These cell lines are therefore expected to be of significance since they should allow us to examine in the future whether GSNO in fact is an intracellular mutagenic species.

7.9. Conclusions

GSH plays a key role in protecting proteins from being *S*-nitrosated by NO_x-donating drugs in Mutatect and HeLa cells. HeLa cells show significantly higher levels of both Pro-SNO and GSNO than Mutatect cells likely due to a 50% lower level of FDH activity. Permanent down-regulation of FDH levels in Mutatect cells by RNA interference and antisense techniques significantly enhanced NO_x-mediated formation of both Pro-SNO and GSNO. This confirmed the role of FDH in protein *S*-nitrosation. A GSH-dependent metabolic cycle involving enzymes FDH and GR that is capable of preventing protein *S*-nitrosation is proposed.

General discussion, significance and conclusions

Work described in this thesis and publication resulting from it (*Appendix A4*) have contributed towards a better understanding of the roles of tumor-infiltrating inflammatory cells and factors derived from them in genetic instability. It has also increased our understanding of the mechanisms of NO_x-mediated genetic instability and post-translational protein modification. The Mutatect system has been demonstrated to be a powerful model for examining these complex processes. Using this mouse model, our laboratory had previously shown that factors in the tumor microenvironment are responsible for an increase frequency of mutation in tumor cells *in vivo* and that these factors may include *neutrophil*-derived ROS/NO_x. Several questions raised in the previous experiments in our laboratory (see *General Introduction*) were answered in this thesis.

Previous attempts to increase neutrophil infiltration by direct intratumoral injection of interleukin-8 were only effective in 8 of 36 tumors, which is believed to be due to inconsistencies associated with interleukin-8 injection. I therefore developed interleukin-8-secreting Mutatect cell lines in an attempt to produce a more consistent source of interleukin-8 in the tumors. These tumors, especially Mutatect TM-28, consistently exhibited high levels of neutrophil infiltration. However, the effect on tumor biology of interleukin-8 expression was found to be complex. High local concentration of interleukin-8 appeared to be anti-tumorigenic, while lower concentrations appeared to give some selective growth advantage to interleukin-8-secreting cells in the tumors. This is of relevance as it may explain some of the paradoxical effects of interleukin-8 described by different workers (121-124). The local concentration of interleukin-8 also appears to have a complex effect on neutrophil infiltration. At high concentrations of interleukin-8, neutrophil levels were only moderately elevated, likely due either to disruption of interleukin-8 concentration-gradient or to anti-tumor effects of neutrophils leading to interleukin-8 gene instability. At very low interleukin-8 concentrations, neutrophil levels were also not very high, likely due to insufficient chemokine. *Intermediate concentrations of interleukin-8 (e.g., in TM-28 tumors) appeared to be "optimal" since neutrophil levels in these tumors were consistently very high.*

Consistent recruitment of high number of neutrophils in tumors allowed us to demonstrate that neutrophils can in fact increase the frequency of mutations in tumors and that there is a strong association between neutrophil-derived ROS/NO_x and a high incidence

of mutations. Most human tumors exhibit some form of chromosomal genetic instability. In addition, many types of solid tumors are infiltrated with inflammatory cells such as neutrophils or macrophages. Indeed, the level of infiltration has been correlated with several parameters associated with tumor progression (see *General Introduction*). Interleukin-8 has been found in many human tumors, and its presence is associated with extensive neutrophil infiltration and poor prognosis (46, 113, 114). Our results show that interleukin-8-producing tumors can have high levels of neutrophil infiltration and this is associated with a high level of genetic instability. I believe this to be very significant and novel finding suggesting that ***genetic instability and the progression of tumors may be, to some extent, a consequence of infiltrating inflammatory cells.*** We have hypothesized that inflammatory cells contribute to genetic instability *via* generating ROS/NO_x into the tumor microenvironment. Inhibition of genetic instability by dietary vitamin E has demonstrated a possible involvement of ROS/NO_x in the process. However, the effects of vitamin E were complicated by its additional effect on neutrophil distribution. High levels of neutrophils also led to a high level of protein nitrotyrosine formation, a marker of reactive NO_x such as ONOO⁻. *The presence of nitrotyrosine in histones implied that these reactive species can enter the nucleus, where they could induce mutations by causing DNA damage.*

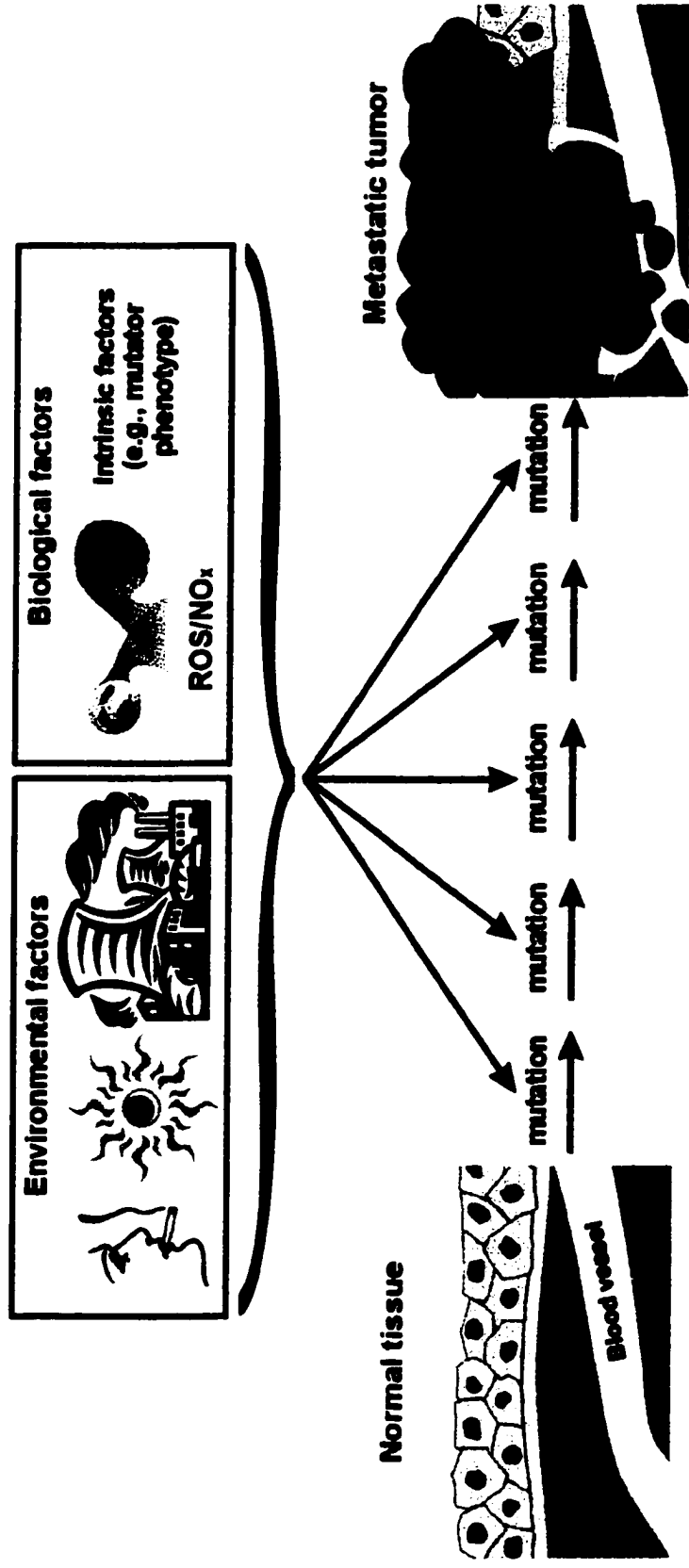
Several unexpected and novel findings were observed using the Mutatetect mouse model. Identification of interleukin-8 transgene instability was significant because it allowed additional insight into the complex biology of tumor development and the role of inflammatory cells. Our proposed mechanism of interleukin-8 transgene instability parallels the accepted mechanism of tumor progression (see *General Introduction*). Both involve genetic damage followed by selective pressure leading to the expansion of clonal populations. The contribution of neutrophil-derived NO_x towards interleukin-8 instability is consistent with their mutagenic role. Unexpectedly, vitamin E had a dramatic effect on neutrophil distribution. This effect of vitamin E suggested that its protective effect is complex and should be taken into account when examining the action of vitamin E in various conditions. In addition, ***using the Mutatetect model, we have found for the first time evidence of a post-translational modification of histones in vivo by NO_x.*** Mass spectrometric techniques were used to identify nitrotyrosine-containing proteins in biological tissues. This modification may be a marker of long-term *in vivo* exposure to reactive NO_x.

species, which have been implicated in many inflammatory disorders including cancer and neurodegenerative disorders. Histones are the most abundant proteins associated with DNA in eukaryotes; post-translational modifications of histones can affect assembly of chromatin and expression of many important genes. Thus, in the research area of histone modification, this may be a very significant finding. It warrants further investigation into the effect of nitrotyrosine modification on chromatin assembly, gene expression and possible inhibition/activation of the other post-translational modifications. In addition, in the research area of inflammatory disorders and cancer, this may also be a very significant finding. Many researchers have described nuclear staining for nitrotyrosine in histological sections of various tissues containing inflammatory cells. Our finding that the nuclear staining likely represents histone modification suggests that those tissues may have been exposed to NO_x for a long time.

In general, our results have produced a better understanding of the role of inflammation in cancer development. Current thinking of the involvement of inflammation in cancer development is that inflammatory cell-derived mutagenic factors induce *initiation* of cancer, since up to one-third of human cancers have a history of chronic inflammation. It has been postulated that inflammation-derived mutagenic factors (mainly ROS/ NO_x) induce the initial mutation in a “mutator gene” (e.g., genes encoding DNA repair enzymes or affecting cell-cycle check-points). Mutations in these genes are believed to predispose cells to accumulation of further mutations (4). However, these mutations do not always increase the frequency of mutations (1) and most tumor cells do not exhibit mutations in mutator genes. Thus, the postulate that inflammation is only involved in the initial process of cancer development may not apply to all cancers. Inflammatory cell infiltration in solid tumors is associated with tumor invasiveness and other parameters of tumor progression, suggesting the involvement of inflammation also in later stages of cancer. Our previous results together with ones presented in this thesis have shown that a high level of inflammatory cell in solid tumors is capable of inducing a high level of genetic instability. Since *multiple* genetic changes are needed to convert a normal cell into a malignant and metastatic cell, the presence of inflammatory cells in established tumors should be seen as a potential contributor to these genetic changes. A fully developed cancer is not likely the result of a single factor; *a combination of many factors, including inflammatory factors,*

FIGURE D.1. Factors contributing to accumulation of mutations in cancer development.

Cancer is not likely a result of one factor. Combination of many factors including environmental factors (e.g., cigarette smoking, ultraviolet radiation, industrial pollution), inflammatory factors (macrophages and neutrophils) and internal biological factors (e.g., mutator phenotype) are all likely to contribute towards the accumulation of mutation in a cancer.



internal factors (e.g., mutator phenotype), as well as environmental factors, are likely to contribute towards the accumulation of mutation in a cancer (Fig. D.1).

Multiple steps in cancer development are characterized by mutational events that confer some form of *selective* growth advantage to the affected cells (1). Mutations that lead to activation of cellular proto-oncogenes or inactivation of tumor-suppressor genes can provide such a selective advantage. We observed a high frequency of mutation at the *hprt* locus in tumor cells *in vivo*, as a consequence of high level of inflammation. Mutations occurring at this locus are used as a *non-selective* surrogate marker of mutations occurring elsewhere in the genome. Thus, a high frequency of mutations at the *hprt* gene suggests that there is an increased probability of a mutation occurring in cellular proto-oncogenes and tumor-suppressor genes. Future experiments could address this questions by comparing of mutations in Mutatect cells *in vivo* and *in vitro* in some cellular proto-oncogenes or tumor-suppressor genes.

In vitro experiments using Mutatect cell proved useful in studying the mechanisms of NO_x-mediated genetic instability and post-translational protein modification. For reasons not yet understood, we were unable to induce mutations in Mutatect cells by NO_x-donating drugs using conditions described previously in our laboratory. However, under these conditions, little intracellular Pro-SNO or GSNO was formed, which is explained by *unusually* high levels of FDH in Mutatect cells growing *in vitro* in complete medium. Recent preliminary results show that the formation of intracellular Pro-SNO correlates with the induction of mutations in the tumor cells. Since levels of Pro-SNO and GSNO are regulated by the same metabolic cycle involving FDH, the association between Pro-SNO and mutagenicity might also implicate GSNO in mutagenicity. However, a role of Pro-SNO in mutagenicity has not been ruled out. For example, S-nitrosation may inhibit DNA-repair enzymes (202), which may further lead to un-repaired DNA damage and consequently induction of mutations. This hypothesis needs to be further examined. We identified 2 conditions that can induce a high level of intracellular RSNO in Mutatect cells. These conditions allowed us to better understand the mechanism of Pro-SNO formation, which is likely different from the mechanism of protein nitrotyrosine formation. These conditions were also useful in understanding the role of FDH in protein S-nitrosation.

Protein S-nitrosation is increasingly becoming recognized as a ubiquitous regulatory reaction, comparable to protein phosphorylation (208). The mechanism of regulation of protein S-nitrosation has not been previously demonstrated. My experiments provide evidence that GSH plays a key role in protecting proteins from being S-nitrosated by NO_x species. We also show specifically that FDH-dependent detoxification of GSNO protects against NO_x-mediated protein S-nitrosation. ***In this thesis I proposed a GSH-dependent metabolic cycle involving FDH and GR that regulates the level of protein S-nitrosation.*** This is expected to have considerable biological significance. The distribution and level of FDH are known to vary among different tissues (86, 206, 207). Until recently, the significance of this variability was considered in terms of its “classical” property, viz., its ability to detoxify formaldehyde. The identification of the novel functions for FDH including detoxification of GSNO and regulation of protein S-nitrosation suggests it may play a key role in the regulation of biological effects of NO_x in cells and tissues. Pro-SNO is also expected to vary in tissues, exposed to NO[•], depending upon their level of FDH.

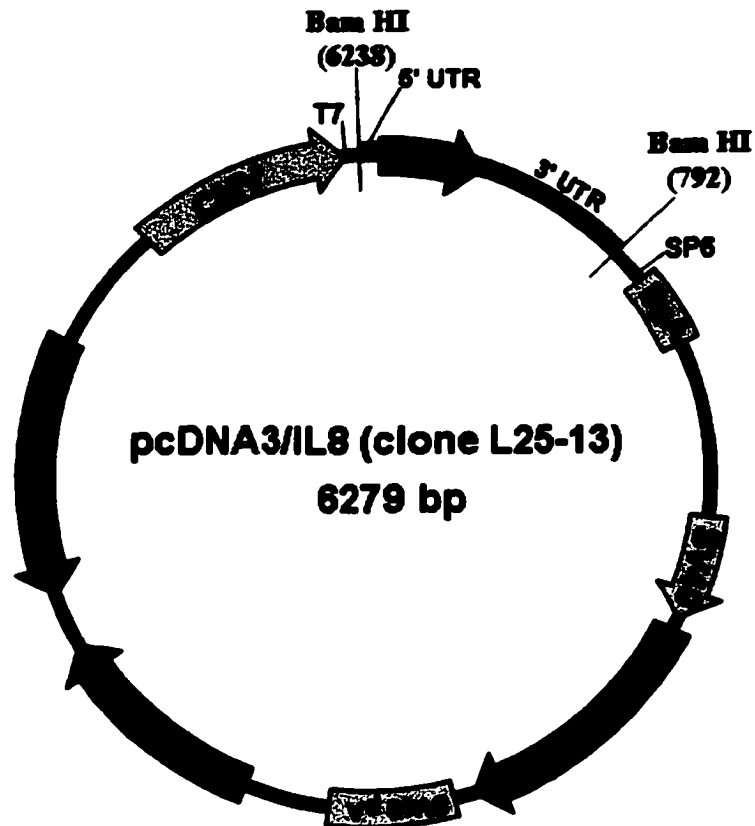
Several new Mutatect cell lines were developed (*Appendix A3*) that should have applications other than those reported in this thesis. Interleukin-8-producing lines, especially TM-28, have been useful in addressing questions that do not form part of this thesis. Recently, TM-28 tumors were used to demonstrate a dramatic difference between α and γ form of dietary tocopherol (vitamin E) in their effects on genetic instability and neutrophil distribution⁷. TM-28 tumors are being currently used to study the effects of other dietary antioxidants. Two Mutatect cell lines (MAG-23 and MG) with altered levels of GSH were also developed. They should be useful for examining the role of GSH levels in NO_x-mediated mutagenicity and S-nitrosation in cultured Mutatect cells. These lines should also be useful for examining the role of GSH levels in mutagenicity *in vivo* in animal experiments. Finally, several Mutatect cell lines with low levels of FDH were developed using antisense and RNA interference techniques. Since a high level of GSNO was shown to be formed in these cells after exposure to NO_x-donating drugs, the level of mutagenicity induced under these conditions should help to demonstrate whether GSNO is indeed an intracellular NO_x carrier. It should also demonstrate the role of FDH in NO_x-mediated mutagenicity.

⁷ Soo CC, Haqqani AS, Hidiroglou N, Parker R, Swanson JE and Birnboim HC, manuscript in preparation.

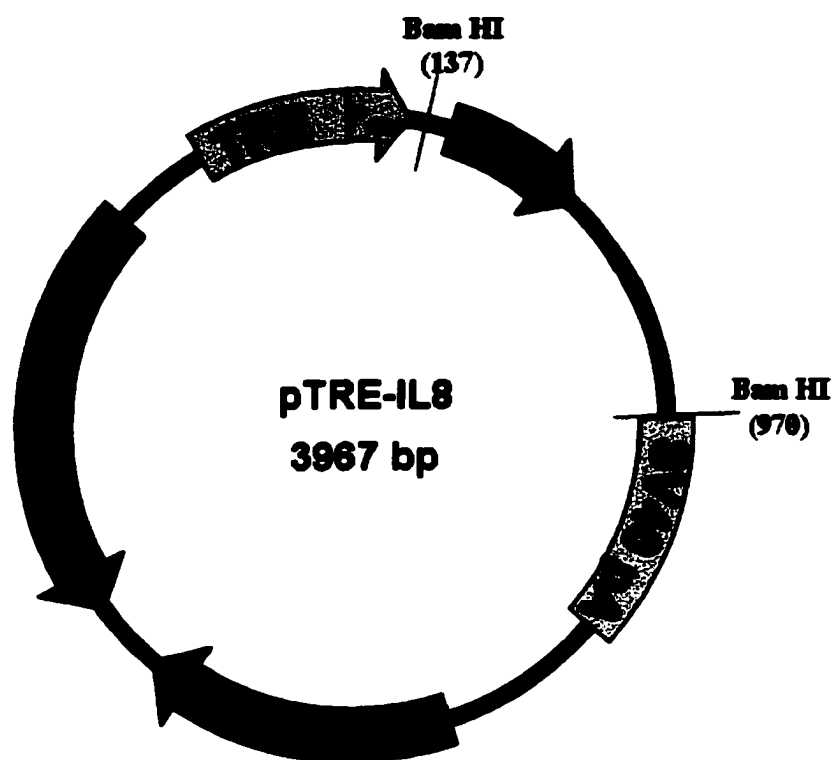
In conclusion, lessons learned from this thesis provide insight into the complexity of cancer development and the contribution of inflammatory cells to mutations and tumor progression. Furthermore, they provide additional insight into other possible roles of ROS/NO_x both *in vitro* and *in vivo*.

Appendices

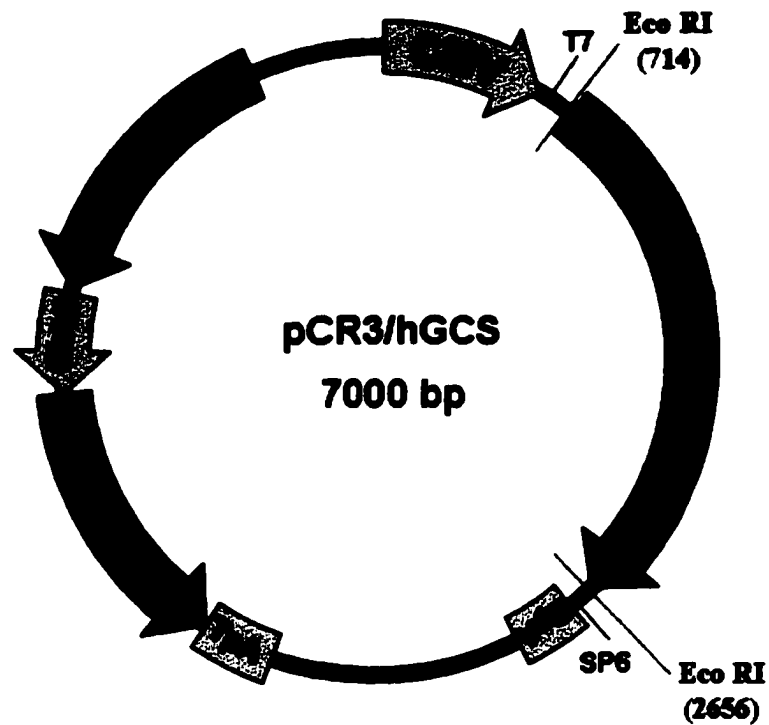
A1: Constructs and plasmids



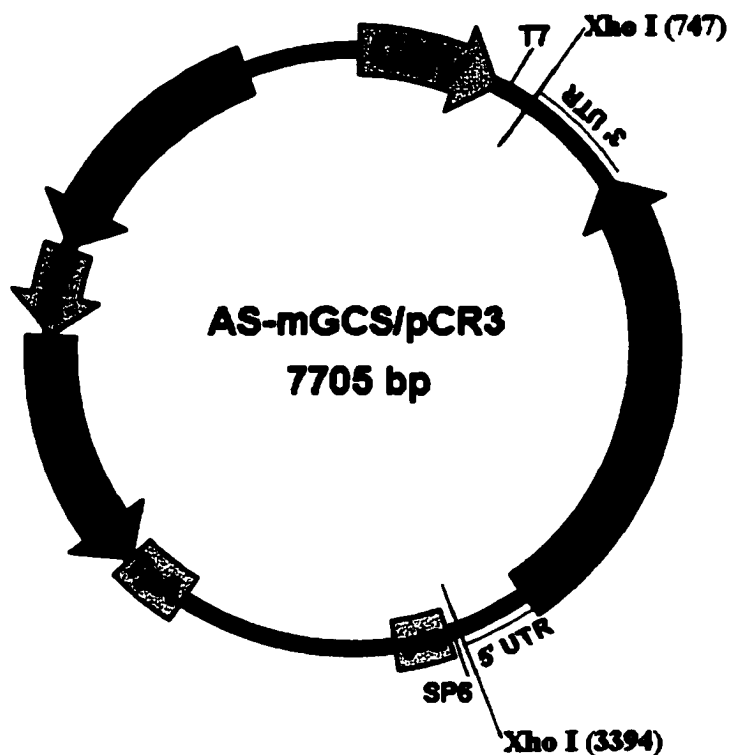
CMV	cytomegalovirus promoter (5539-6193)
T7	T7 promoter (6194-6212)
IL-8	human interleukin-8 coding region (9-308) obtained from IMAGE clone 328322
5'UTR	partial 5' untranslated region of interleukin-8 (6270-8)
3'UTR	partial 3' untranslated region of interleukin-8 (309-790)
SP6	SP6 promoter (883-900)
BpA	poly A signal from bovine growth hormone (902-1133)
SV40	Simian virus-40 promoter (1674-1999)
Neomycin	neomycin-resistant gene (2035-2829)
SV40 pA	poly A signal from SV40 (2884-3256)
ColEI	origin of replication (3516-4189)



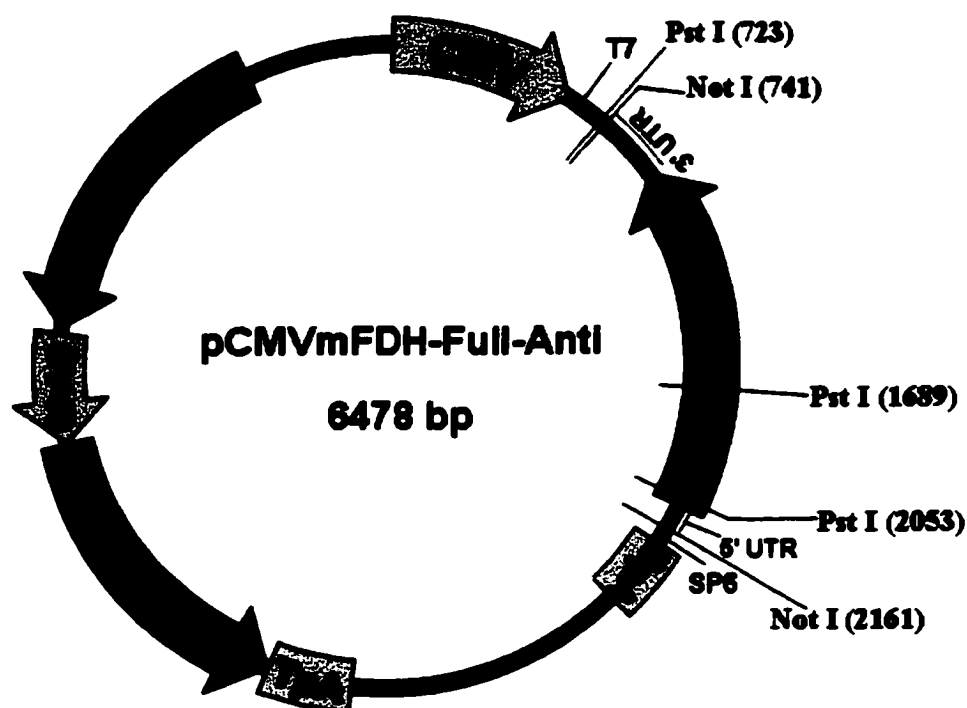
TRE	tTA response elements (3634-3951)
P_{min}	minimal cytomegalovirus promoter (3952-103)
IL-8	human interleukin-8 coding region (187-486) obtained from pcDNA3/IL8
SV40 pA	poly A signal from SV40 (975-1432)
ColE1	origin of replication (1782-2425)
Ampicillin	ampicillin-resistant gene (2573-3433)



CMV	cytomegalovirus promoter (1-596)
T7	T7 promoter (638-657)
hGCS	human γ-glutamylcysteine synthase coding region (730-2643)
SP6	SP6 promoter (2716-2733)
BpA	poly A signal from bovine growth hormone (2738-2966)
TpA	poly A signal from thymidine kinase (3865-4136)
Kan/Neo	kanamycin and neomycin-resistant gene (4137-5133)
SV40	Simian virus-40 promoter (5134-5491)
Ampicillin	ampicillin resistant gene (5510-6541)



CMV	cytomegalovirus promoter (1-596)
T7	T7 promoter (638-657)
3'UTR	3' untranslated region of mGCS (747-1187)
mGCS	mouse γ -glutamylcysteine synthase coding region (1188-3101) obtained from IMAGE clone 1970400
5'UTR	5' untranslated region of mGCS (3102-3393)
SP6	SP6 promoter (3421-3438)
BpA	poly A signal from bovine growth hormone (3443-3671)
TpA	poly A signal from thymidine kinase
Kan/Neo	kanamycin and neomycin-resistant gene (4842-5838)
SV40	Simian virus-40 promoter (5839-6196)
Ampicillin	ampicillin resistant gene (6215-7246)



CMV	cytomegalovirus promoter (1-596)
T7	T7 promoter (638-657)
3'UTR	3' untranslated region of mFDH (747-956)
mFDH	mouse formaldehyde dehydrogenase coding region (957-2081) obtained from IMAGE clone 2812535
5'UTR	5' untranslated region of mFDH (2082-2153)
SP6	SP6 promoter (2194-2211)
BpA	poly A signal from bovine growth hormone (2216-2444)
TpA	poly A signal from thymidine kinase (3343-3614)
Kan/Neo	kanamycin and neomycin-resistant gene (3615-4611)
SV40	Simian virus-40 promoter (4612-4969)
Ampicillin	ampicillin resistant gene (4988-6019)

A2: An enhanced electrophoretic transfer of histones for western blot analysis

A2.1. Summary

Using standard western blotting procedures, we encountered inconsistent results while analyzing for nitrotyrosine-containing cellular proteins. This was due to inefficient transfer of many proteins from polyacrylamide gel to polyvinylidene difluoride membrane. The majority of the low molecular weight proteins were histones, which had a transfer efficiency of <10%. We describe here a modified and simple procedure to improve their transfer. The polyacrylamide gel and the filter papers near the cathode side of the gel were equilibrated with an sodium dodecyl sulfate-containing buffer for a short period of time prior to the transfer, and the transfer was carried out in a pH 10 buffer. With these two modification, a dramatic improvement (>95% efficiency) in the transfer of histones was observed. More effective transfer of many other proteins was also seen. We believe that this procedure will be useful for analyzing post-translational modification of histones by western blot analysis.

A2.2. Introduction

In western blotting procedures, proteins are first separated by polyacrylamide gel electrophoresis (PAGE) (209) and then electrophoretically transferred to a nitrocellulose or polyvinylidene difluoride (PVDF) membrane for immunoblotting (210). In the PAGE step, sodium dodecyl sulfate (SDS) is usually included in the gel and the running buffer to “anionize” the proteins (i.e., give them a strong negative charge) so that they can travel towards the anode independent of their negative charge and shape. However, in the electrophoretic transfer step, SDS is usually omitted since it can interfere with the binding of proteins to membrane. Part of the SDS in the polyacrylamide gel is removed by pre-equilibration with an SDS-free solution prior to electrophoretic transfer. Using this standard transfer procedure, we found that several low molecular weight (MW) proteins, mainly histones, failed to adequately electrotransfer from polyacrylamide gel to PVDF membrane, leading to inconsistent estimates of these proteins by western blotting. We now describe a modification of the electrotransfer procedure that considerably increases the efficiency of transfer of these proteins and greatly improves the reliability of analysis by western blotting.

A2.3. Materials and methods

Protein samples and SDS-PAGE analysis. Protein extracts of Mutatect tumors were isolated by homogenization and sonication, as described elsewhere in *chapter 5*. The homogenate was diluted in 2 % SDS, 10 % glycerol, 1 % 2-mercaptoethanol, 0.0005 % bromophenol blue, 125 mM MOPS, pH 6.8 and heated at 100°C for 5-10 min. Fluorescamine was used to quantify protein (147). Samples were separated by 12% discontinuous SDS-PAGE (0.75 mm thick) using the Laemmli's method (209) and electrophoretically transferred to PVDF membranes (Millipore, Canada) using either the standard or the enhanced method as described below.

Standard electrotransfer of proteins. Prior to transfer, each component of the electrophoretic transfer sandwich (Fig. A2.1; pads, filter papers, polyacrylamide gel and PVDF membrane) was pre-equilibrated for > 15 min in the transfer buffer-8.3 (3 mM Na₂CO₃, 10 mM NaHCO₃, 10% methanol, pH 8.3) or buffer-10 (20 mM sodium carbonate, 10% methanol, pH 10). The sandwich was assembled as shown in Fig. A2.1 and the electrophoretic transfer carried out at 15 V for 18 h. Transferred proteins were detected by staining the membrane with Ponceau S, while the non-transferred proteins were detected by staining the residual gel with Coomassie blue.

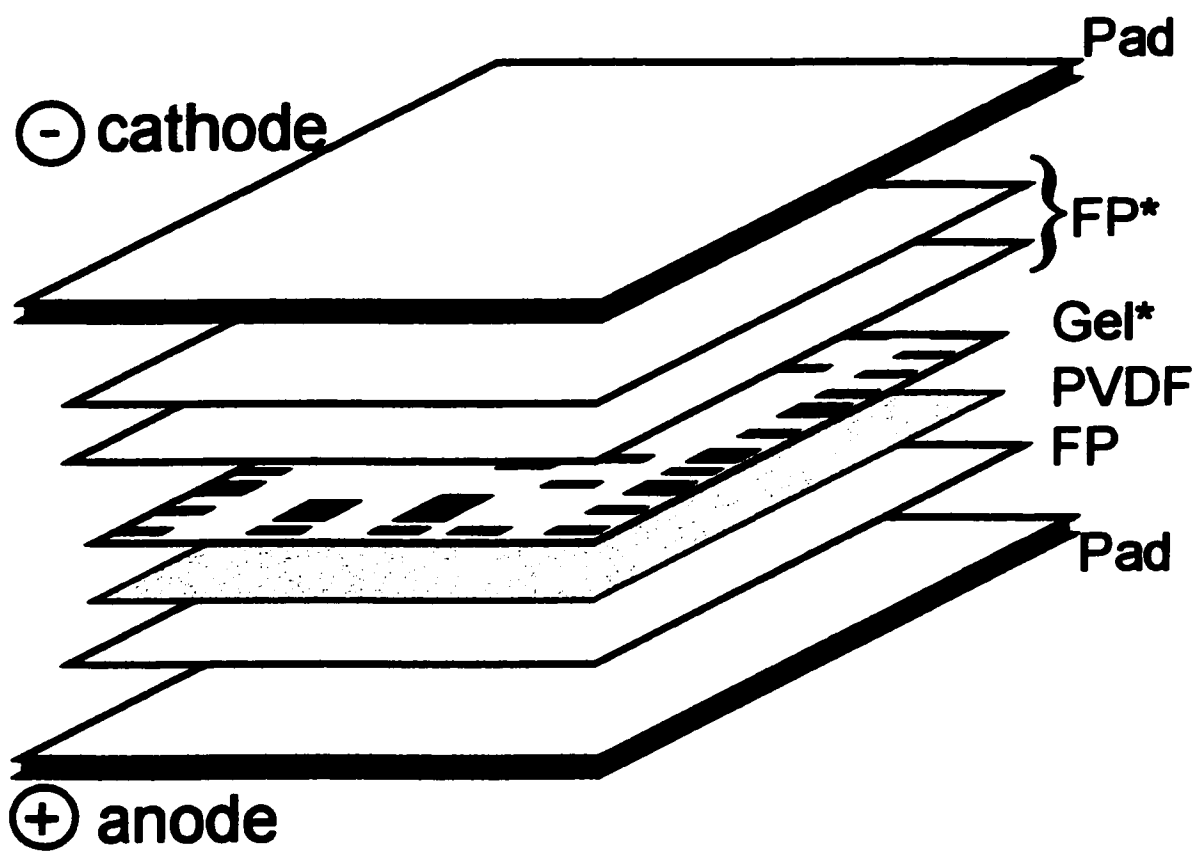
Enhanced electrotransfer of proteins. Prior to transfer, the polyacrylamide gel and the filter papers on the cathode side of the gel (Fig. A2.1) were soaked in an SDS-containing buffer (buffer-10 plus 0.2 % SDS) for > 15 min. Other components in the electrophoretic transfer sandwich (pads, remaining filters and PVDF membrane) were incubated in buffer-10 for > 15 min. The sandwich was assembled as shown in Fig. A2.1 and the electrophoretic transfer carried out at 15 V for 18 h. Transferred proteins were detected by staining the membrane with Ponceau S, while the non-transferred proteins were detected by staining the residual gel with Coomassie blue.

A2.4. Results and Discussion

The most commonly used procedure for electrophoretic transfer of proteins from polyacrylamide gels to PVDF membranes is carried out in a pH 8.3 buffer. Using these conditions, we found inconsistencies while analyzing for nitrotyrosine-containing proteins by western blotting (Fig. A2.2A), which was due to their poor transfer. Protein bands showing

FIGURE A2.1. Electrophoretic transfer sandwich.

The sandwich consists of two pads, three filter papers (FP), a polyacrylamide gel and a PVDF membrane. An asterisk (*) suggest that the components (gel and the indicated FP) are pre-equilibrated in SDS-containing buffer. The rest of the materials is pre-equilibrated in the transfer buffer. The PVDF membrane was hydrated in 100% methanol prior to use. See text for details.



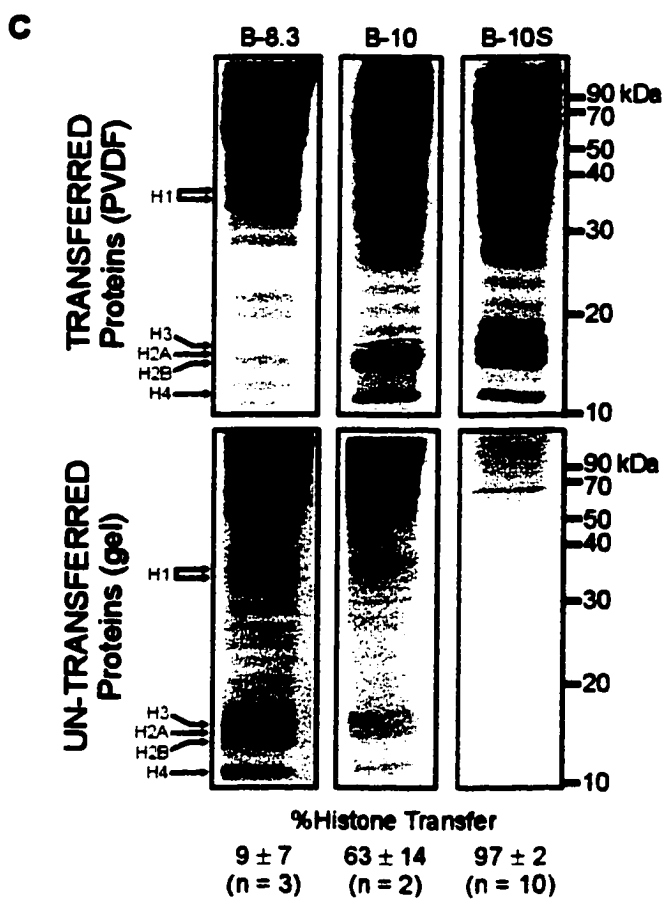
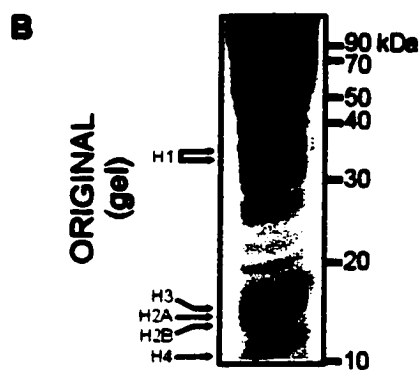
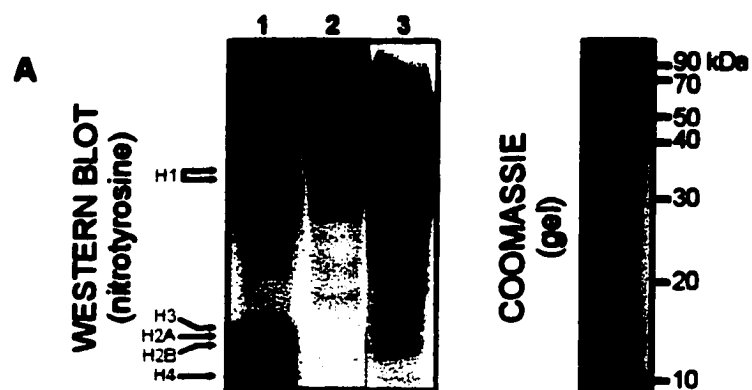
the most inconsistencies on western blots were low MW, which were eventually identified as being histones (*chapter 5*). To explore the lack of histone transfer, we analyzed tumor extracts by PAGE, then analyzed proteins before and after transfer. Only about 5-15 % of the histones was found to transfer to PVDF membrane (Fig. A2.2B and C). Since histones are very basic proteins (i.e., $pI \geq 10$), we postulated that the lack of the transfer was due to their high positive charge (i.e., incomplete deprotonation) at pH 8.3. In order to give them a more negative charge (i.e., deprotonate them), we increased the pH of the transfer buffer. About 60 % of histones transferred to PVDF membrane within 20 min at pH 10 (Fig A2.2). Maintaining electrotransfer for longer than 20 min, however, caused no additional histones to transfer (data not shown). Thus, although increasing the pH improved the transfer, it was insufficient to provide sufficient negative charge required for complete transfer.

We therefore tested whether SDS, a strongly anionic detergent that binds tightly to proteins could provide the additional negative charge to histones to aid in their complete transfer. Since an excess of SDS can interfere with protein binding to the hydrophobic membrane, different methods of exposure to SDS were tested. The simplest and most suitable procedure proved to be the following. Prior to transfer, only the polyacrylamide gel and filter paper on the cathode side of the PVDF membrane were equilibrated in 0.2% SDS buffer (see Methods). Other components of the sandwich apparatus were equilibrated in buffer lacking SDS (Fig. A2.1). The transfer was carried out at pH 10. With this procedure, >95% of the histones were found to transfer to the PVDF membrane within 20 min. In addition, the transfer of many other proteins, including high MW proteins, was also dramatically increased. Thus, inclusion of a small amount of SDS “upstream” of the gel was sufficient to maintain the negative charge of proteins long enough to dramatically improve the electrotransfer process.

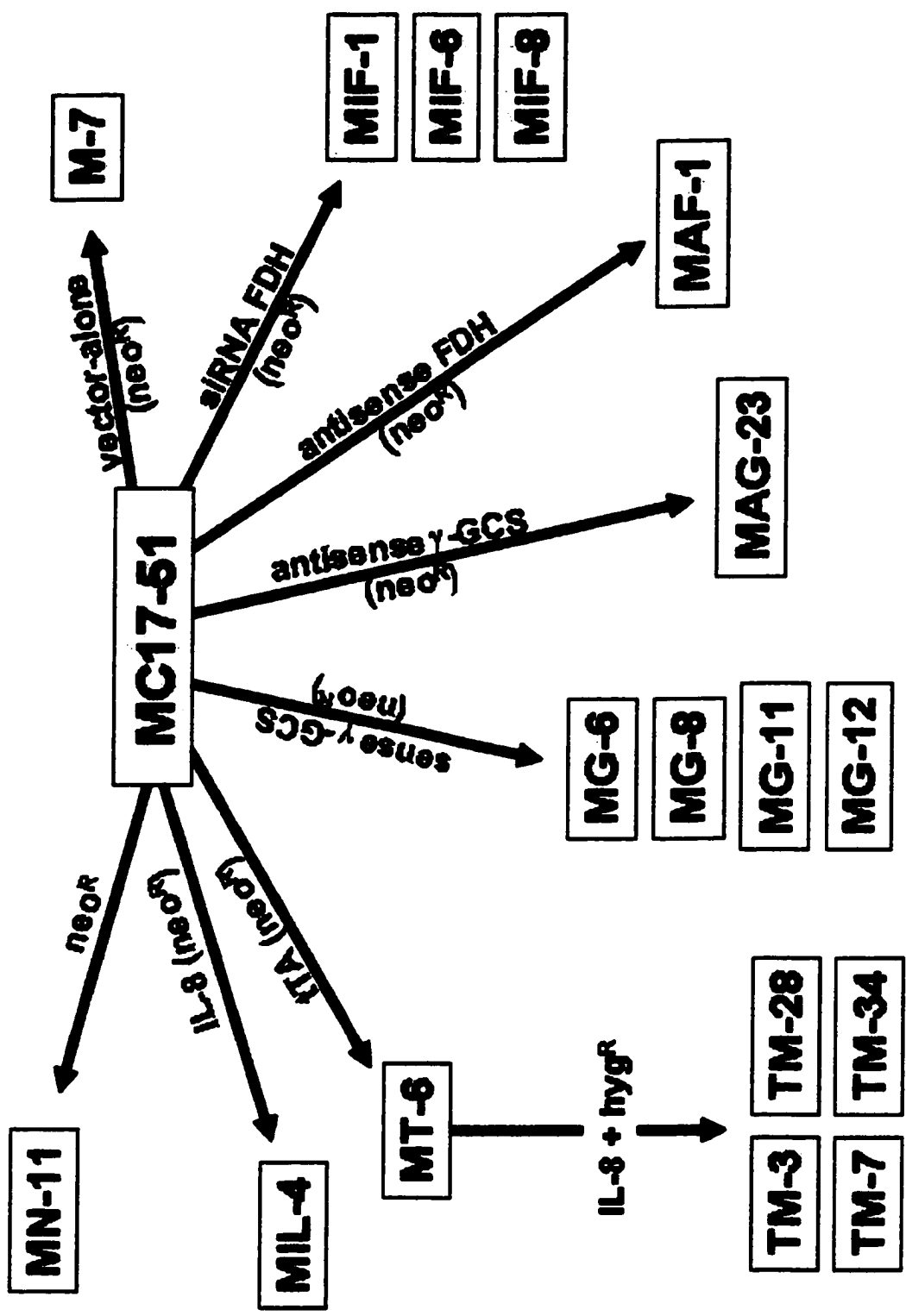
This transfer method was useful for producing consistent results for the study of nitrotyrosine-modification of histones in Mutatect experiments (*chapter 5*) and other experiments (unpublished data). We believe that this transfer procedure should also be useful in analyzing other post-translational modifications of histones by western blotting. In addition, the method may also be useful in analyzing other proteins (either basic or high MW proteins) that fail to transfer under standard conditions.

FIGURE A2.2. Enhancement in electrotransfer of histones.

A) Inconsistencies observed in western blotting using anti-nitrotyrosine antibody using standard transfer procedure. *Lanes 1-3* represent 3 independent blots containing the same tumor protein extract (25 µg/lane). Coomassie blue staining of the extract is also shown. Note that histones appear on western blots under these conditions. **B, C)** Comparison of various transfer procedures. Protein extracts from Mutatect cells were analyzed by SDS-PAGE on several lanes (30 µg/lane). The gel was cut into 4 set of lanes. One set was Coomassie blue-stained to estimate the total amount of protein (Original). The other 3 sets were transferred to PVDF membranes using buffer pH 8.3 (B-8.3), pH 10 (B-10) or pH 10 plus SDS-treatment (B-10S) as described in Materials and Methods. After the transfer, the PVDF membranes were Ponceu S-stained to detect the transferred proteins, and the residual gels were Coomassie blue-stained to detect the untransferred protein. Other details in Materials and Methods.



A3: Mutatect cell lines



A4: Papers and Publications

Following are the manuscripts published in refereed journals that were a direct result of my Ph.D research and are mostly reported in *chapter 1* to *5* of the thesis.

1. **Haqqani AS, Sandhu JK, and Birnboim HC.** (2001). Constitutive expression of interleukin-8 by Mutatect cells markedly affects their tumor biology. Carcinogenesis 22: 243-250.
2. **Haqqani AS, Sandhu JK, and Birnboim HC.** (2000). Expression of interleukin-8 promotes neutrophil infiltration and genetic instability in Mutatect tumors. Neoplasia 2: 561-568.
3. **Haqqani AS, Sandhu JK, and Birnboim HC.** (1999). A myeloperoxidase-specific assay based upon bromide-dependent chemiluminescence of luminol. Analytical Biochemistry. 273: 126-132.
4. **Haqqani AS, Sandhu JK, and Birnboim HC.** (2002). Dietary vitamin E affects neutrophil distribution and genetic instability in murine Mutatect tumors. Nutrition and Cancer 42: 105-111.
5. **Sandhu JK, Haqqani AS, and Birnboim HC.** (2000). Effect of dietary vitamin E on spontaneous or nitric oxide donor-induced mutations in a mouse tumor model. Journal of National Cancer Institute. 92: 1429-1433.
6. **Haqqani AS, Kelly JF, and Birnboim HC.** (2002). Selective nitration of histone tyrosine residues *in vivo* in Mutatect tumors. Journal of Biological Chemistry. 277: 3614-21.

Most of the results from *chapter 6* and *7* are currently being written up for a manuscript (*authors*: Haqqani AS, Do HK and Birnboim HC). Results from *Appendix A2* are also currently being written up for a short report (*authors*: Haqqani AS and Birnboim HC). Following are the titles of poster abstracts presented at various international or national meetings that were a result of the research conducted in this Ph.D. thesis.

1. **Haqqani AS, Kelly JF, and Birnboim HC** (2002). Tyrosine residues are selectively nitrated in Mutatect tumors. *Ottawa Health Research Institute Scientific Research Symposium*, 1, 14, Ottawa, ON, Canada.
2. **Haqqani AS and Birnboim HC** (2001). Genetic manipulation of glutathione levels in Mutatect mouse tumor cells affects nitric oxide induced cytotoxicity. *Proceedings of the American Association for Cancer Research*, 42, 887, New Orleans, LA.
3. **Haqqani AS, Kelly JF, and Birnboim HC** (2001). Nitrotyrosine (Ntyr) residues in histones as a potential marker of chronic nitric oxide (NO) exposure. *Canadian Federation of Biological Societies*, 44, F043, Ottawa, ON, Canada.
4. **Haqqani AS and Birnboim HC** (2000). Interleukin-8-producing tumor cells are selectively eliminated in the Mutatect mouse tumor model. *Proceedings of the American Association for Cancer Research*, 41, 5274, San Francisco, CA.
5. **Haqqani AS, Grant DG, and Birnboim HC** (2000). Interleukin-8 expression by Mutatect tumours: Effect of dietary vitamin E on mutagenesis mediated by neutrophil-

- derived reactive nitrogen species. *Canadian Federation of Biological Societies*, 43, F060, Ottawa, ON, Canada.
6. **Haqqani AS**, Sandhu JK, and Birnboim HC (1999). Mutatect, a mouse model to study the mutagenic factors present in a tumor microenvironment. *Prediction of tumor response to therapy: Molecular markers and the microenvironment*, p24, October 8-9, Montreal, QC, Canada.
 7. Do HK, **Haqqani AS**, and Birnboim HC (2002). S-nitrosothiol formation and mutagenesis in cultured Mutatect cells after exposure to various nitric oxide donating compounds. *Ottawa Health Research Institute Scientific Research Symposium*, 1, 49, Ottawa, ON, Canada.
 8. Soo CC, **Haqqani AS**, and Birnboim HC (2002). Effects of dietary vitamin E on mutations in the mouse Mutatect tumors. *Ottawa Health Research Institute Scientific Research Symposium*, 1, 1, Ottawa, ON, Canada.
 9. Kelly JF, **Haqqani AS**, and Birnboim HC (2001). Towards the development of a mass spectrometry method that selectively detects nitration of tyrosine in proteins. *Bionorth 2001: Annual Ottawa Life Sciences International Conference and Exhibition*, 8, Ottawa, ON, Canada.

References

Reference List

1. Coleman WB, Tsongalis GJ (2002). Role of genomic instability in the development of human cancer. *In: The molecular basis of human cancer*. Editors: Coleman WB, Tsongalis GJ. New Jersey: Humana Press. pp. 115-142.
2. Reinartz JJ (2002). Cancer genes. *In: The molecular basis of human cancer*. Editors: Coleman WB, Tsongalis GJ. New Jersey: Humana Press. pp. 45-64.
3. Miller JH, Miller JH, Suzuki DT, Lewontin RC, Gelbart WM (2000). Cancer as a genetic disease. *In: An Introduction to genetic analysis*. New York: W.H. Freeman and Co.
4. Jackson AL, Loeb LA (2001). The contribution of endogenous sources of DNA damage to the multiple mutations in cancer. *Mutat. Res.* **477**: 7-21.
5. Thomas S, Lowe JE, Knowles RG, Green IC, Green MH (1998). Factors affecting the DNA damaging activity of superoxide and nitric oxide. *Mutat. Res.* **402**: 77-84.
6. Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L (2000). Neutrophils: molecules, functions and pathophysiological aspects. *Lab. Invest.* **80**: 617-653.
7. Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P, Pritchard KA, Jr. (1998). Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc. Natl. Acad. Sci. U. S. A* **95**: 9220-9225.
8. Xia Y, Dawson VL, Dawson TM, Snyder SH, Zweier JL (1996). Nitric oxide synthase generates superoxide and nitric oxide in arginine- depleted cells leading to peroxynitrite-mediated cellular injury. *Proc. Natl. Acad. Sci. U. S. A* **93**: 6770-6774.
9. Xia Y, Zweier JL (1997). Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. *Proc. Natl. Acad. Sci. U. S. A* **94**: 6954-6958.
10. Kanofsky JR, Wright J, Miles-Richardson GE, Tauber AI (1984). Biochemical requirements for singlet oxygen production by purified human myeloperoxidase. *J. Clin. Invest.* **74**: 1489-1495.
11. Burney S, Caulfield JL, Niles JC, Wishnok JS, Tannenbaum SR (1999). The chemistry of DNA damage from nitric oxide and peroxynitrite. *Mutat. Res.* **424**: 37-49.
12. Ducrocq C, Blanchard B, Pignatelli B, Ohshima H (1999). Peroxynitrite: an endogenous oxidizing and nitrating agent. *Cell Mol. Life Sci.* **55**: 1068-1077.
13. Wiseman H, Halliwell B (1996). Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochem. J.* **313** (Pt 1): 17-29.

14. Stuehr DJ (1996). Purification and properties of nitric oxide synthases. *Meth. Enzymol.* **268**: 324-333.
15. Heck DE (2001). *NO, RSNO, ONOO-, NO+, *NOO, NOx--dynamic regulation of oxidant scavenging, nitric oxide stores, and cyclic GMP-independent cell signaling. *Antioxid. Redox. Signal.* **3**: 249-260.
16. Stamler JS, Singel DJ, Loscalzo J (1992). Biochemistry of nitric oxide and its redox-activated forms. *Science* **258**: 1898-1902.
17. Ignarro LJ (1996). Physiology and pathophysiology of nitric oxide. *Kidney Int.* **49 Suppl.** **55**: S2-S5.
18. Ignarro LJ (1991). Heme-dependent activation of guanylate cyclase by nitric oxide: A novel signal transduction mechanism. *Blood Vessels* **28**: 67-73.
19. Hogg N (2002). The biochemistry and physiology of S-nitrosothiols. *Annu. Rev. Pharmacol. Toxicol.* **42**: 585-600.
20. Stamler JS, Lamas S, Fang FC (2001). Nitrosylation. the prototypic redox-based signaling mechanism. *Cell* **106**: 675-683.
21. Stamler JS (1994). Redox signaling: Nitrosylation and related target interactions of nitric oxide. *Cell* **78**: 931-936.
22. Felley-Bosco E (1998). Role of nitric oxide in genotoxicity: implication for carcinogenesis. *Cancer Metastasis Rev.* **17**: 25-37.
23. Grant DD, Goldstein R, Karsh J, Birnboim HC (2001). Nitric oxide donors induce large-scale deletion mutations in human lymphoblastoid cells: implications for mutations in T-lymphocytes from arthritis patients. *Environ. Mol. Mutagen.* **38**: 261-267.
24. Viera L, Ye YZ, Estevez AG, Beckman JS (1999). Immunohistochemical methods to detect nitrotyrosine. *Methods Enzymol.* **301**: 373-381.
25. Ischiropoulos H (1998). Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. *Arch. Biochem. Biophys.* **356** : 1-11.
26. Yermilov V, Rubio J, Becchi M, Friesen MD, Pignatelli B, Ohshima H (1995). Formation of 8-nitroguanine by the reaction of guanine with peroxynitrite in vitro. *Carcinogenesis* **16**: 2045-2050.
27. Van der Vliet A, Eiserich JP, Kaur H, Cross CE, Halliwell B (1996). Nitrotyrosine as biomarker for reactive nitrogen species. *Methods Enzymol.* **269**: 175-184.

28. Eiserich JP, Hristova M, Cross CE, Jones AD, Freeman BA, Halliwell B, Van der Vliet A (1998). Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* **391**: 393-397.
29. Sakaguchi AA, Miura S, Takeuchi T, Hokari R, Mizumori M, Yoshida H, Higuchi H, Mori M, Kimura H, Suzuki H, Ishii H (1999). Increased expression of inducible nitric oxide synthase and peroxynitrite in *Helicobacter pylori* gastric ulcer. *Free Radic. Biol. Med.* **27**: 781-789.
30. Greis KD, Zhu S, Matalon S (1996). Identification of nitration sites on surfactant protein A by tandem electrospray mass spectrometry. *Arch. Biochem. Biophys.* **335**: 396-402.
31. Cassina AM, Hodara R, Souza JM, Thomson L, Castro L, Ischiropoulos H, Freeman BA, Radi R (2000). Cytochrome c nitration by peroxynitrite. *J. Biol. Chem.* **275**: 21409-21415.
32. Zhu S, Basiouny KF, Crow JP, Matalon S (2000). Carbon dioxide enhances nitration of surfactant protein A by activated alveolar macrophages. *Am. J. Physiol. Lung Cell Mol. Physiol.* **278**: L1025-L1031.
33. Eiserich JP, Estevez AG, Bamberg TV, Ye YZ, Chumley PH, Beckman JS, Freeman BA (1999). Microtubule dysfunction by posttranslational nitrotyrosination of alpha-tubulin: a nitric oxide-dependent mechanism of cellular injury. *Proc. Natl. Acad. Sci. USA* **96**: 6365-6370.
34. Ames BN, Shigenaga MK, Hagen TM (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. USA* **90**: 7915-7922.
35. Maeda H, Akaike T (1998). Nitric oxide and oxygen radicals in infection, inflammation, and cancer. *Biochemistry (Mosc)* **63**: 854-865.
36. Li CQ, Pignatelli B, Ohshima H (2001). Increased oxidative and nitrative stress in human stomach associated with cagA+ *Helicobacter pylori* infection and inflammation. *Dig. Dis. Sci.* **46**: 836-844.
37. Heeringa P, Bijl M, Jager-Krikken A, Zandvoort A, Dijkstra G, Moshage H, Tervaert JW, Tiebosch AT, Kallenberg CG, van Goor H (2001). Renal expression of endothelial and inducible nitric oxide synthase, and formation of peroxynitrite-modified proteins and reactive oxygen species in Wegener's granulomatosis. *J. Pathol.* **193**: 224-232.
38. Kaminsky DA, Mitchell J, Carroll N, James A, Soultanakis R, Janssen Y (1999). Nitrotyrosine formation in the airways and lung parenchyma of patients with asthma. *J. Allergy Clin. Immunol.* **104**: 747-754.

39. Hansen PR, Holm AM, Svendsen UG, Olsen PS, Andersen CB (2000). Apoptosis and formation of peroxynitrite in the lungs of patients with obliterative bronchiolitis. *J. Heart Lung Transplant.* **19**: 160-166.
40. Kaur H, Halliwell B (1994). Evidence for nitric oxide-mediated oxidative damage in chronic inflammation. Nitrotyrosine in serum and synovial fluid from rheumatoid patients. *FEBS Lett.* **350**: 9-12.
41. Mapp PI, Klocke R, Walsh DA, Chana JK, Stevens CR, Gallagher PJ, Blake DR (2001). Localization of 3-nitrotyrosine to rheumatoid and normal synovium. *Arthritis Rheum.* **44**: 1534-1539.
42. Garcia-Monzon C, Majano PL, Zubia I, Sanz P, Apolinario A, Moreno-Otero R (2000). Intrahepatic accumulation of nitrotyrosine in chronic viral hepatitis is associated with histological severity of liver disease. *J. Hepatol.* **32**: 331-338.
43. Oates JC, Christensen EF, Reilly CM, Self SE, Gilkeson GS (1999). Prospective measure of serum 3-nitrotyrosine levels in systemic lupus erythematosus: correlation with disease activity. *Proc. Assoc. Am. Physicians* **111**: 611-621.
44. Saleh D, Ernst P, Lim S, Barnes PJ, Giaid A (1998). Increased formation of the potent oxidant peroxynitrite in the airways of asthmatic patients is associated with induction of nitric oxide synthase: effect of inhaled glucocorticoid. *FASEB J.* **12**: 929-937.
45. Nowacki MP, Janik P, Nowacki PM (1996). Inflammation and metastases. *Med. Hypotheses* **47**: 193-196.
46. Bellocq A, Antoine M, Flahault A, Philippe C, Crestani B, Bernaudin JF, Mayaud C, Milleron B, Baud L, Cadranel J (1998). Neutrophil alveolitis in bronchioloalveolar carcinoma: induction by tumor-derived interleukin-8 and relation to clinical outcome. *Am. J. Pathol.* **152**: 83-92.
47. Kaffenberger W, Clasen BP, van Beuningen D (1992). The respiratory burst of neutrophils, a prognostic parameter in head and neck cancer? *Clin. Immunol. Immunopathol.* **64**: 57-62.
48. Mazzone A, Fioravanti A, Pasotti D, Ricevuti G (1988). Neutrophil defects are a prognostic factor in acute myeloid leukemia. *Haematologica* **73**: 293-296.
49. Timoshenko AV, Andre S, Kayser K, Gabius HJ (2001). Lectin (WGA)-dependent superoxide anion release by neutrophils as a prognostic factor in lung cancer. *Anticancer Res.* **21**: 3453-3456.
50. Arenberg DA, Keane MP, DiGiovine B, Kunkel SL, Strom SR, Burdick MD, Iannettoni MD, Strieter RM (2000). Macrophage infiltration in human non-small-cell lung cancer: the role of CC chemokines. *Cancer Immunol. Immunother.* **49**: 63-70.

51. Torisu H, Ono M, Kiryu H, Furue M, Ohmoto Y, Nakayama J, Nishioka Y, Sone S, Kuwano M (2000). Macrophage infiltration correlates with tumor stage and angiogenesis in human malignant melanoma: possible involvement of TNF α and IL-1 α . *Int. J. Cancer* **85**: 182-188.
52. Fossati G, Ricevuti G, Edwards SW, Walker C, Dalton A, Rossi ML (1999). Neutrophil infiltration into human gliomas. *Acta Neuropathol. (Berl)* **98**: 349-354.
53. Yamakawa Y, Fujimura M, Hidaka T, Yasoshima K, Saito S (1999). Inflammatory malignant fibrous histiocytoma of the ovary producing interleukin-6: A case report. *Gynecol. Oncol.* **75**: 484-489.
54. Ono M, Torisu H, Fukushi J, Nishie A, Kuwano M (1999). Biological implications of macrophage infiltration in human tumor angiogenesis. *Cancer Chemother. Pharmacol.* **43 Suppl**: S69-S71.
55. Etoh T, Shibuta K, Barnard GF, Kitano S, Mori M (2000). Angiogenin expression in human colorectal cancer: the role of focal macrophage infiltration. *Clin. Cancer Res.* **6**: 3545-3551.
56. Leek RD, Hunt NC, Landers RJ, Lewis CE, Royds JA, Harris AL (2000). Macrophage infiltration is associated with VEGF and EGFR expression in breast cancer. *J. Pathol.* **190**: 430-436.
57. Leek RD, Landers RJ, Harris AL, Lewis CE (1999). Necrosis correlates with high vascular density and focal macrophage infiltration in invasive carcinoma of the breast. *Br. J. Cancer* **79**: 991-995.
58. Leek RD, Lewis CE, Whitehouse R, Greenall M, Clarke J, Harris AL (1996). Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. *Cancer Res.* **56**: 4625-4629.
59. Irani J, Goujon JM, Ragni E, Peyrat L, Hubert J, Saint F, Mottet N (1999). High-grade inflammation in prostate cancer as a prognostic factor for biochemical recurrence after radical prostatectomy. Pathologist Multi Center Study Group. *Urology* **54**: 467-472.
60. Goto T, Haruma K, Kitadai Y, Ito M, Yoshihara M, Sumii K, Hayakawa N, Kajiyama G (1999). Enhanced expression of inducible nitric oxide synthase and nitrotyrosine in gastric mucosa of gastric cancer patients. *Clin. Cancer Res.* **5**: 1411-1415.
61. Mendes RV, Martins AR, De Nucci G, Murad F, Soares FA (2001). Expression of nitric oxide synthase isoforms and nitrotyrosine immunoreactivity by B-cell non-Hodgkin's lymphomas and multiple myeloma. *Histopathology* **39**: 172-178.

62. Kojima M, Morisaki T, Tsukahara Y, Uchiyama A, Matsunari Y, Mibu R, Tanaka M (1999). Nitric oxide synthase expression and nitric oxide production in human colon carcinoma tissue. *J. Surg. Oncol.* **70**: 222-229.
63. Vickers SM, MacMillan-Crow LA, Green M, Ellis C, Thompson JA (1999). Association of increased immunostaining for inducible nitric oxide synthase and nitrotyrosine with fibroblast growth factor transformation in pancreatic cancer. *Arch. Surg.* **134**: 245-251.
64. Ekmekcioglu S, Ellerhorst J, Smid CM, Prieto VG, Munsell M, Buzaid AC, Grimm EA (2000). Inducible nitric oxide synthase and nitrotyrosine in human metastatic melanoma tumors correlate with poor survival. *Clin. Cancer Res.* **6**: 4768-4775.
65. Ohshima H, Bartsch H (1994). Chronic infections and inflammatory processes as cancer risk factors: Possible role of nitric oxide in carcinogenesis. *Mutat. Res. Fundam. Mol. Mech. Mutagen.* **305**: 253-264.
66. Wilkinson D, Sandhu JK, Breneman JW, Tucker JD, Birnboim HC (1995). *Hprt* mutants in a transplantable murine tumour arise more frequently *in vivo* than *in vitro*. *Br. J. Cancer* **72**: 1234-1240.
67. Tamir S, Tannenbaum SR (1996). The role of nitric oxide (NO.) in the carcinogenic process. *Biochim. Biophys. Acta Rev. Cancer* **1288**: F31-F36.
68. Wink DA, Vodovotz Y, Laval J, Laval F, Dewhirst MW, Mitchell JB (1998). The multifaceted roles of nitric oxide in cancer. *Carcinogenesis* **19**: 711-721.
69. Reynolds TY, Rockwell S, Glazer PM (1996). Genetic instability induced by the tumor microenvironment. *Cancer Res.* **56**: 5754-5757.
70. Sandhu JK, Privora HF, Wenckebach G, Birnboim HC (2000). Neutrophils, nitric oxide synthase, and mutations in the Mutatect murine tumor model. *Am. J. Pathol.* **156**: 509-518.
71. Lu SC (1999). Regulation of hepatic glutathione synthesis: current concepts and controversies. *FASEB J.* **13**: 1169-1183.
72. Luperchio S, Tamir S, Tannenbaum SR (1996). NO-induced oxidative stress and glutathione metabolism in rodent and human cells
11. *Free Radic. Biol. Med.* **21** : 513-519.
73. Walker MW, Kinter MT, Roberts RJ, Spitz DR (1995). Nitric oxide-induced cytotoxicity: involvement of cellular resistance to oxidative stress and the role of glutathione in protection
7. *Pediatr. Res.* **37**: 41-49.

74. Romao PR, Fonseca SG, Hothersall JS, Noronha-Dutra AA, Ferreira SH, Cunha FQ (1999). Glutathione protects macrophages and *Leishmania major* against nitric oxide-mediated cytotoxicity. *Parasitology* **118**: 559-566.
75. Ibi M, Sawada H, Kume T, Katsuki H, Kaneko S, Shimohama S, Akaike A (1999). Depletion of intracellular glutathione increases susceptibility to nitric oxide in mesencephalic dopaminergic neurons. *J. Neurochem.* **73**: 1696-1703.
76. Naito Y, Yoshikawa T, Boku Y, Fujii T, Masui Y, Tanaka Y, Fujita N, Yoshida N, Kondo M (2000). Protective role of intracellular glutathione against nitric oxide-induced necrosis in rat gastric mucosal cells. *Aliment. Pharmacol. Ther.* **14 Suppl 1**:145-52.: 145-152.
77. Birnboim HC, Privora H (2000). Depletion of Intracellular Glutathione Reduces Mutations by Nitric Oxide-Donating Drugs. *Nitric Oxide* **4**: 496-504.
78. Michiels C, Raes M, Toussaint O, Remacle J (1994). Importance of Se-glutathione peroxidase, catalase, and Cu/Zn-SOD for cell survival against oxidative stress. *Free Radic. Biol. Med.* **17**: 235-248.
79. Meister A, Anderson ME (1983). Glutathione. *Annu. Rev. Biochem.* **52**: 711-760.
80. Uotila L, Koivusalo M (1974). Formaldehyde dehydrogenase from human liver. Purification, properties, and evidence for the formation of glutathione thiol esters by the enzyme. *J Biol. Chem.* **249**: 7653-7663.
81. Dicker E, Cederbaum AI (1984). Effect of acetaldehyde and cyanamide on the metabolism of formaldehyde by hepatocytes, mitochondria, and soluble supernatant from rat liver. *Arch Biochem Biophys* **232**: 179-188.
82. Danielsson O, Shafqat J, Estonius M, Jornvall H (1994). Alcohol dehydrogenase class III contrasted to class I. Characterization of the cyclostome enzyme, the existence of multiple forms as for the human enzyme, and distant cross-species hybridization. *Eur. J Biochem* **225**: 1081-1088.
83. Estonius M, Hoog JO, Danielsson O, Jornvall H (1994). Residues specific for class III alcohol dehydrogenase. Site-directed mutagenesis of the human enzyme. *Biochemistry* **33**: 15080-15085.
84. Uotila L, Koivusalo M (1997). Expression of formaldehyde dehydrogenase and S-formylglutathione hydrolase activities in different rat tissues. *Adv. Exp. Med. Biol.* **414**:365-71.: 365-371.
85. Deltour L, Foglio MH, Duester G (1999). Metabolic deficiencies in alcohol dehydrogenase *Adh1*, *Adh3*, and *Adh4* null mutant mice. Overlapping roles of *Adh1* and *Adh4* in ethanol clearance and metabolism of retinol to retinoic acid. *J Biol. Chem.* **274**: 16796-16801.

86. Hedberg JJ, Hoog JO, Nilsson JA, Xi Z, Elfving A, Grafstrom RC (2000). Expression of alcohol dehydrogenase 3 in tissue and cultured cells from human oral mucosa. *Am. J. Pathol.* **157**: 1745-1755.
87. Grafstrom RC (1990). In vitro studies of aldehyde effects related to human respiratory carcinogenesis. *Mutat. Res.* **238**: 175-184.
88. Wink DA, Beckman JS, Ford PC (1996). Kinetics of nitric oxide reaction in liquid and gas phase. *In: Methods in nitric oxide research*. Editors: Feelisch M, Stamler JS. Toronto: John Wiley & Sons, Inc. pp. 29-37.
89. Lipton AJ, Johnson MA, Macdonald T, Lieberman MW, Gozal D, Gaston B (2001). S-nitrosothiols signal the ventilatory response to hypoxia. *Nature* **413**: 171-174.
90. Liu L, Hausladen A, Zeng M, Que L, Heitman J, Stamler JS (2001). A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. *Nature* **410**: 490-494.
91. Brigelius-Flohé R, Traber MG (1999). Vitamin E: function and metabolism. *FASEB J.* **13**: 1145-1155.
92. Burton GW (1994). Vitamin E: molecular and biological function. *Proc. Nutr. Soc.* **53**: 251-262.
93. Kamal-Eldin A, Appelqvist LA (1996). The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids* **31**: 671-701.
94. Patterson RE, White E, Kristal AR, Neuhouser ML, Potter JD (1997). Vitamin supplements and cancer risk: the epidemiologic evidence. *Cancer Causes and Control* **8** : 786-802.
95. Wang Y, Ichiba M, Oishi H, Iyadomi M, Shono N, Tomokuni K (1997). Relationship between plasma concentrations of beta-carotene and alpha-tocopherol and life-style factors and levels of DNA adducts in lymphocytes. *Nutrition & Cancer* **27**: 69-73.
96. Albanes D, Heinonen OP, Huttunen JK, Taylor PR, Virtamo J, Edwards BK, Haapakoski J, Rautalahti M, Hartman AM, Palmgren J, et al (1995). Effects of alpha-tocopherol and beta-carotene supplements on cancer incidence in the Alpha-Tocopherol Beta-Carotene Cancer Prevention Study. *Am. J. Clin. Nutr.* **62**: 1427S-1430S.
97. Rautalahti MT, Virtamo JR, Taylor PR, Heinonen OP, Albanes D, Haukka JK, Edwards BK, Kärkkäinen PA, Stolzenberg-Solomon RZ, Huttunen J (1999). The effects of supplementation with alpha-tocopherol and beta-carotene on the incidence and mortality of carcinoma of the pancreas in a randomized, controlled trial. *Cancer* **86**: 37-42.
98. Young KJ, Lee PN (1999). Intervention studies on cancer. *Eur. J. Cancer Prev.* **8** : 91-103.

99. Zhang S, Hunter DJ, Forman MR, Rosner BA, Speizer FE, Colditz GA, Manson JE, Hankinson SE, Willett WC (1999). Dietary carotenoids and vitamins A, C, and E and risk of breast cancer. *J. Natl. Cancer Inst.* **91**: 547-556.
100. Eichholzer M, Stähelin HB, Lüdlin E, Bernasconi F (1999). Smoking, plasma vitamins C, E, retinol, and carotene, and fatal prostate cancer: seventeen-year follow-up of the prospective Basel study. *Prostate* **38**: 189-198.
101. Woodson K, Tangrea JA, Barrett MJ, Virtamo J, Taylor PR, Albanes D (1999). Serum alpha-tocopherol and subsequent risk of lung cancer among male smokers. *J. Natl. Cancer Inst.* **91**: 1738-1743.
102. Jacobs EJ, Connell CJ, Patel AV, Chao A, Rodriguez C, Seymour J, McCullough ML, Calle EE, Thun MJ (2001). Vitamin C and vitamin E supplement use and colorectal cancer mortality in a large American Cancer Society cohort. *Cancer Epidemiol. Biomarkers Prev.* **10**: 17-23.
103. Virtamo J, Edwards BK, Virtanen M, Taylor PR, Malila N, Albanes D, Huttunen JK, Hartman AM, Hietanen P, Maenpää H, Koss L, Nordling S, Heinonen OP (2000). Effects of supplemental alpha-tocopherol and beta-carotene on urinary tract cancer: incidence and mortality in a controlled trial (Finland). *Cancer Causes Control* **11**: 933-939.
104. Helzlsouer KJ, Huang HY, Alberg AJ, Hoffman S, Burke A, Norkus EP, Morris JS, Comstock GW (2000). Association Between alpha-Tocopherol, gamma-Tocopherol, Selenium, and Subsequent Prostate Cancer. *J. Natl. Cancer Inst.* **92**: 2018-2023.
105. Gann PH, Ma J, Giovannucci E, Willett W, Sacks FM, Hennekens CH, Stampfer MJ (1999). Lower prostate cancer risk in men with elevated plasma lycopene levels: results of a prospective analysis. *Cancer Res.* **59**: 1225-1230.
106. Brawley OW, Parnes H (2000). Prostate cancer prevention trials in the USA. *Eur. J. Cancer* **36**: 1312-1315.
107. Sandhu JK, Haqqani AS, Birnboim HC (2000). Effect of dietary vitamin E on spontaneous or nitric oxide donor-induced mutations in a mouse tumor model. *J. Natl. Cancer Inst.* **92**: 1429-1433.
108. Sandhu JK, Birnboim HC (1997). Mutagenicity and cytotoxicity of reactive oxygen and nitrogen species in the MN-11 murine tumor cell line. *Mutat. Res.* **379**: 241-252.
109. Belperio JA, Keane MP, Arenberg DA, Addison CL, Ehlert JE, Burdick MD, Strieter RM (2000). CXC chemokines in angiogenesis
1. *J. Leukoc. Biol.* **68**: 1-8.
110. Singh RK, Varney ML, Bucana CD, Johansson SL (1999). Expression of interleukin-8 in primary and metastatic malignant melanoma of the skin. *Melanoma Res.* **9**: 383-387.

111. Singh RK, Gutman M, Radinsky R, Bucana CD, Fidler IJ (1994). Expression of interleukin 8 correlates with the metastatic potential of human melanoma cells in nude mice. *Cancer Res.* **54**: 3242-3247.
112. Colasante A, Mascetra N, Brunetti M, Lattanzio G, Diodoro M, Caltagirone S, Musiani P, Aiello FB (1997). Transforming growth factor beta 1, interleukin-8 and interleukin-1, in non-small-cell lung tumors. *Am. J. Respir. Crit. Care Med.* **156**: 968-973.
113. Eisma RJ, Spiro JD, Kreutzer DL (1999). Role of angiogenic factors: coexpression of interleukin-8 and vascular endothelial growth factor in patients with head and neck squamous carcinoma. *Laryngoscope* **109**: 687-693.
114. Kitadai Y, Haruma K, Sumii K, Yamamoto S, Ue T, Yokozaki H, Yasui W, Ohmoto Y, Kajiyama G, Fidler IJ, Tahara E (1998). Expression of interleukin-8 correlates with vascularity in human gastric carcinomas. *Am. J. Pathol.* **152**: 93-100.
115. Sakamoto K, Masuda T, Mita S, Ishiko T, Nakashima Y, Arakawa H, Egami H, Harada S, Matsushima K, Ogawa M (1992). Interleukin-8 is constitutively and commonly produced by various human carcinoma cell lines. *Int. J. Clin. Lab. Res.* **22**: 216-219.
116. Galffy G, Mohammed KA, Dowling PA, Nasreen N, Ward MJ, Antony VB (1999). Interleukin 8: an autocrine growth factor for malignant mesothelioma. *Cancer Res.* **59**: 367-371.
117. Van Meir E, Ceska M, Effenberger F, Walz A, Grouzmann E, Desbaillets I, Frei K, Fontana A, De Tribolet N (1992). Interleukin-8 is produced in neoplastic and infectious diseases of the human central nervous system. *Cancer Res.* **52**: 4297-4305.
118. Desbaillets I, Diserens AC, Tribolet N, Hamou MF, Van Meir EG (1997). Upregulation of interleukin 8 by oxygen-deprived cells in glioblastoma suggests a role in leukocyte activation, chemotaxis, and angiogenesis. *J. Exp. Med.* **186**: 1201-1212.
119. Rot A (1991). Chemotactic potency of recombinant human neutrophil attractant/activation protein-1 (interleukin-8) for polymorphonuclear leukocytes of different species. *Cytokine* **3**: 21-27.
120. Sugawara T, Miyamoto M, Takayama S, Kato M (1995). Separation of neutrophils from blood in human and laboratory animals and comparison of the chemotaxis. *J. Pharmacol. Toxicol. Methods* **33**: 91-100.
121. Hirose K, Hakozaiki M, Nyunoya Y, Kobayashi Y, Matsushita KXTT, Mikata A, Mukaida N, Matsushima K (1995). Chemokine gene transfection into tumour cells reduced tumorigenicity in nude mice in association with neutrophilic infiltration. *Br. J. Cancer* **72**: 708-714.

122. Zhang H, Wei S, Sun J, Coppola D, Zhong B, Wu GD, Goodwin B, Sebti S, Djeu JY, Blanck G (2000). Retinoblastoma protein activation of interleukin 8 expression inhibits tumor cell survival in nude mice. *Cell Growth Differ.* 11: 635-639.
123. Lejeune P, Reisser D, Onier N, Lagadec P, Lindley I, Jeannin JF (1994). Interleukin-8 has antitumor effects in the rat which are not associated with polymorphonuclear leukocyte cytotoxicity. *Cancer Immunol. Immunother.* 38: 167-170.
124. Kitadai Y, Takahashi Y, Haruma K, Naka K, Sumii K, Yokozaki H, Yasui W, Mukaida N, Ohmoto Y, Kajiyama G, Fidler IJ, Tahara E (1999). Transfection of interleukin-8 increases angiogenesis and tumorigenesis of human gastric carcinoma cells in nude mice. *Br. J. Cancer* 81: 647-653.
125. Yoshida A, Yoshida S, Khalil AK, Ishibashi T, Inomata H (1998). Role of NF-kappaB-mediated interleukin-8 expression in intraocular neovascularization. *Invest Ophthalmol. Vis. Sci.* 39: 1097-1106.
126. Yuan A, Yang PC, Yu CJ, Chen WJ, Lin FY, Kuo SH, Luh KT (2000). Interleukin-8 messenger ribonucleic acid expression correlates with tumor progression, tumor angiogenesis, patient survival, and timing of relapse in non-small-cell lung cancer. *Am. J. Respir. Crit Care Med.* 162: 1957-1963.
127. Lennon G, Auffray C, Polymeropoulos M, Soares MB (1996). The I.M.A.G.E. Consortium: an integrated molecular analysis of genomes and their expression. *Genomics* 33: 151-152.
128. Cowling RT, Chaim Birnboim H (1994). Incorporation of [³²P]orthophosphate into inorganic polyphosphates by human granulocytes and other human cell types. *J. Biol. Chem.* 269: 9480-9485.
129. Mellane KM, Kraemer R, Smith B (1985). Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. *J. Pharmacol. Methods* 14: 157-167.
130. Allan G, Bhattacharjee P, Brook CD, Read NG, Parke AJ (1985). Myeloperoxidase activity as a quantitative marker of polymorphonuclear leukocyte accumulation into an experimental myocardial infarct--the effect of ibuprofen on infarct size and polymorphonuclear leukocyte accumulation. *J. Cardiovasc. Pharmacol.* 7: 1154-1160.
131. Schierwagen C, Bylund-Fellenius AC, Lundberg C (1990). Improved method for quantification of tissue PMN accumulation measured by myeloperoxidase activity. *J. Pharmacol. Methods* 23: 179-186.
132. Barone FC, Hillegass LM, Price WJ, White RF, Lee EV, Feuerstein GZ, Sarau HM, Clark RK, Griswold DE (1991). Polymorphonuclear leukocyte infiltration into cerebral focal ischemic tissue: myeloperoxidase activity assay and histologic verification. *J. Neurosci. Res.* 29: 336-345.

133. Bradley PP, Priebat DA, Christensen RD, Rothstein G (1982). Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J. Invest. Dermatol.* **78**: 206-209.
134. Davies B, Edwards SW (1992). Chemiluminescence of human bloodstream monocytes and neutrophils: an unusual oxidant(s) generated by monocytes during the respiratory burst. *J. Biolumin. Chemilumin.* **7**: 229-238.
135. Jung TW, Peterhans E (1988). Change in the chemiluminescence reactivity pattern during in vitro differentiation of human monocytes to macrophages. *Blut* **56**: 213-220.
136. Mathy-Hartert M, Deby-Dupont G, Melin P, Lamy M, Deby C (1996). Bactericidal activity against *Pseudomonas aeruginosa* is acquired by cultured human monocyte-derived macrophages after uptake of myeloperoxidase. *Experientia* **52**: 167-174.
137. Suzuki K, Ota H, Sasagawa S, Sakatani T, Fujikura T (1983). Assay method for myeloperoxidase in human polymorphonuclear leukocytes. *Anal. Biochem.* **132**: 345-352.
138. Cramer R, Soranzo MR, Dri P, Menegazzi R, Pitotti A, Zabucchi G, Patriarca P (1984). A simple reliable assay for myeloperoxidase activity in mixed neutrophil-eosinophil cell suspensions: application to detection of myeloperoxidase deficiency. *J. Immunol. Methods* **70**: 119-125.
139. Bos A, Wever R, Roos D (1978). Characterization and quantification of the peroxidase in human monocytes. *Biochim. Biophys. Acta* **525**: 37-44.
140. Dahlgren C, Stendahl O (1983). Role of myeloperoxidase in luminol-dependent chemiluminescence of polymorphonuclear leukocytes. *Infect. Immun.* **39**: 736-741.
141. DeChatelet LR, Long GD, Shirley PS, Bass DA, Thomas MJ, Henderson FW, Cohen MS (1982). Mechanism of the luminol-dependent chemiluminescence of human neutrophils. *J. Immunol.* **129**: 1589-1593.
142. Hasegawa H, Suzuki K, Nakaji S, Sugawara K (1997). Analysis and assessment of the capacity of neutrophils to produce reactive oxygen species in a 96-well microplate format using lucigenin- and luminol-dependent chemiluminescence. *J. Immunol. Methods* **210**: 1-10.
143. Grisham MB, Benoit JN, Granger DN (1990). Assessment of leukocyte involvement during ischemia and reperfusion of intestine. *Meth. Enzymol.* **186**: 729-742.
144. Allen RC, Stjernholm RL, Steele RH (1972). Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. *Biochem. Biophys. Res. Commun.* **47**: 679-684.

145. Allen RC (1975). Halide dependence of the myeloperoxidase-mediated antimicrobial system of the polymorphonuclear leukocyte in the phenomenon of electronic excitation. *Biochem. Biophys. Res. Commun.* **63**: 675-683.
146. Kanofsky JR, Hoogland H, Wever R, Weiss SJ (1988). Singlet oxygen production by human eosinophils. *J. Biol. Chem.* **263**: 9692-9696.
147. Udenfriend S, Stein S, Bohlen P, Dairman W, Leimgruber W, Weigle M (1972). Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. *Science* **178**: 871-872.
148. De Vouge MW, Yamazaki A, Bennett SAL, Chen J-H, Shwed PS, Couture C, Birnboim HC (1994). Immunoselection of GRP94/endoplasmic reticulum protein from a KNRK cell-specific lambda gt10 library using antibodies directed against a putative heparanase amino-terminal peptide. *Int. J. Cancer* **56**: 286-294.
149. Sakamaki K, Tomonaga M, Tsukui K, Nagata S (1989). Molecular cloning and characterization of a chromosomal gene for human eosinophil peroxidase. *J. Biol. Chem.* **264**: 16828-16836.
150. Brestel EP (1985). Co-oxidation of luminol by hypochlorite and hydrogen peroxide implications for neutrophil chemiluminescence. *Biochem. Biophys. Res. Commun.* **126**: 482-488.
151. Vilim V, Wilhelm J (1989). What do we measure by a luminol-dependent chemiluminescence of phagocytes? *Free Radic. Biol. Med.* **6**: 623-629.
152. Singh VP, Saluja AK, Bhagat L, van Acker GJ, Song AM, Soltoff SP, Cantley LC, Steer ML (2001). Phosphatidylinositol 3-kinase-dependent activation of trypsinogen modulates the severity of acute pancreatitis
37. J. Clin. Invest **108**: 1387-1395.
153. Varga SM, Beckman NA, Chu M, Braciale TJ (2002). Sensitive detection and quantitation of mouse eosinophils in tissues using an enzymatic eosinophil peroxidase assay: its use to rapidly measure pulmonary eosinophilia during experimental respiratory syncytial virus infection of mice
38. J. Immunol. Methods **262**: 111-120.
154. Birnboim HC, Wilkinson D, Sandhu JK, McLean JR, Ross W (1999). Mutatect: a mouse tumour model for detecting radiation-induced mutations in vivo. *Mutat. Res.* **430**: 275-280.
155. Haqqani AS, Sandhu JK, Birnboim HC (1999). A myeloperoxidase-specific assay based upon bromide-dependent chemiluminescence of luminol. *Anal. Biochem.* **273**: 126-132.
156. Birnboim HC (1993). Extraction of high molecular weight RNA and DNA from cultured mammalian cells. *Meth. Enzymol.* **216**: 154-160.

157. Kettritz R, Gaido ML, Haller H, Luft FC, Jennette CJ, Falk RJ (1998). Interleukin-8 delays spontaneous and tumor necrosis factor- α - mediated apoptosis of human neutrophils. *Kidney Int.* **53**: 84-91.
158. Vytasek R (1982). A sensitive fluorometric assay for the determination of DNA. *Anal. Biochem.* **120**: 243-248.
159. Formigli L, Ibba ML, Tani A, Gandini E, Adembri C, Pratesi C, Novelli GP, Zecchi OS (1997). Vitamin E prevents neutrophil accumulation and attenuates tissue damage in ischemic-reperfused human skeletal muscle. *Histol. Histopathol.* **12**: 663-669.
160. Islam KN, Devaraj S, Jialal I (1998). alpha-Tocopherol enrichment of monocytes decreases agonist-induced adhesion to human endothelial cells. *Circulation* **98**: 2255-2261.
161. Yoshikawa T, Yoshida N, Manabe H, Terasawa Y, Takemura T, Kondo M (1998). alpha-Tocopherol protects against expression of adhesion molecules on neutrophils and endothelial cells. *Biofactors* **7**: 15-19.
162. Erl W, Weber C, Wardemann C, Weber PC (1997). alpha-Tocopheryl succinate inhibits monocytic cell adhesion to endothelial cells by suppressing NF-kappa B mobilization. *Am. J. Physiol.* **273**: H634-H640.
163. Nakamura T, Goto M, Matsumoto A, Tanaka I (1998). Inhibition of NF-kappa B transcriptional activity by alpha-tocopheryl succinate. *Biofactors* **7**: 21-30.
164. Zapolska-Downar D, Zapolski-Downar A, Markiewski M, Ciechanowicz A, Kaczmarczyk M, Naruszewicz M (2000). Selective inhibition by alpha-tocopherol of vascular cell adhesion molecule-1 expression in human vascular endothelial cells. *Biochem. Biophys. Res. Commun.* **274**: 609-615.
165. Marra F, DeFranco R, Grappone C, Parola M, Milani S, Leonarduzzi G, Pastacaldi S, Wenzel UO, Pinzani M, Dianzani MU, Laffi G, Gentilini P (1999). Expression of monocyte chemotactic protein-1 precedes monocyte recruitment in a rat model of acute liver injury, and is modulated by vitamin E. *J. Investig. Med.* **47**: 66-75.
166. Yoshikawa T, Yoshida N (2000). Vitamin E and leukocyte-endothelial cell interactions. *Antioxid. Redox. Signal.* **2**: 821-825.
167. Azzi A, Breyer I, Feher M, Pastori M, Ricciarelli R, Spycher S, Staffieri M, Stocker A, Zimmer S, Zingg JM (2000). Specific cellular responses to alpha-tocopherol. *J. Nutr.* **130**: 1649-1652.
168. Jialal I, Devaraj S, Kaul N (2001). The effect of alpha-tocopherol on monocyte proatherogenic activity. *J. Nutr.* **131**: 389S-394S.
169. Teunissen CE, Markerink-van Ittersum M, de Bruijn C, Steinbusch HW, De Vente J (2002). Evaluation of 3-nitrotyrosine as a marker for 3-nitropropionic acid- induced

oxidative stress in Lewis and Wistar rats and strain-specific whole brain spheroid cultures. *Brain Res.* **931**: 5-20.

170. Vaziri ND, Ni Z, Oveisi F, Liang K, Pandian R (2002). Enhanced nitric oxide inactivation and protein nitration by reactive oxygen species in renal insufficiency. *Hypertension* **39**: 135-141.
171. Beckmann JS, Ye YZ, Anderson PG, Chen J, Accavitti MA, Tarpey MM, White R (1994). Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. *Biol. Chem. Hoppe Seyler* **375**: 81-88.
172. Hogg N, Kalyanaraman B (1999). Nitric oxide and lipid peroxidation. *Biochim. Biophys. Acta* **1411**: 378-384.
173. MacMillan-Crow LA, Crow JP, Kerby JD, Beckman JS, Thompson JA (1996). Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. *Proc. Natl. Acad. Sci. U. S. A* **93**: 11853-11858.
174. Zou MH, Leist M, Ullrich V (1999). Selective nitration of prostacyclin synthase and defective vasorelaxation in atherosclerotic bovine coronary arteries. *Am. J. Pathol.* **154**: 1359-1365.
175. MacMillan-Crow LA, Greendorfer JS, Vickers SM, Thompson JA (2000). Tyrosine nitration of c-SRC tyrosine kinase in human pancreatic ductal adenocarcinoma. *Arch. Biochem. Biophys.* **377**: 350-356.
176. Mihm MJ, Coyle CM, Schanbacher BL, Weinstein DM, Bauer JA (2001). Peroxynitrite induced nitration and inactivation of myofibrillar creatine kinase in experimental heart failure. *Cardiovasc. Res.* **49**: 798-807.
177. Pignatelli B, Li CQ, Boffetta P, Chen Q, Ahrens W, Nyberg F, Mukeria A, Briske-Hohlfeld I, Fortes C, Constantinescu V, Ischiropoulos H, Ohshima H (2001). Nitrated and oxidized plasma proteins in smokers and lung cancer patients. *Cancer Res.* **61**: 778-784.
178. Zhu S, Ware LB, Geiser T, Matthay MA, Matalon S (2001). Increased levels of nitrate and surfactant protein a nitration in the pulmonary edema fluid of patients with acute lung injury. *Am. J. Respir. Crit Care Med.* **163**: 166-172.
179. Giasson BI, Duda JE, Murray IV, Chen Q, Souza JM, Hurtig HI, Ischiropoulos H, Trojanowski JQ, Lee VM (2000). Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. *Science* **290**: 985-989.
180. Gole MD, Souza JM, Choi I, Hertkorn C, Malcolm S, Foust RF, III, Finkel B, Lanken PN, Ischiropoulos H (2000). Plasma proteins modified by tyrosine nitration in acute respiratory distress syndrome. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **278**: L961-L967.

181. Marcondes S, Turko IV, Murad F (2001). Nitration of succinyl-CoA:3-oxoacid CoA-transferase in rats after endotoxin administration. *Proc. Natl. Acad. Sci. U. S. A* **98**: 7146-7151.
182. Ye YZ, Strong M, Huang ZQ, Beckman JS (1996). Antibodies that recognize nitrotyrosine. *Methods Enzymol.* **269**: 201-209.
183. Chazotte-Aubert L, Hainaut P, Ohshima H (2000). Nitric oxide nitrates tyrosine residues of tumor-suppressor p53 protein in MCF-7 cells. *Biochem. Biophys. Res. Commun.* **267**: 609-613.
184. Buchczyk DP, Briviba K, Hartl FU, Sies H (2000). Responses to peroxynitrite in yeast: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a sensitive intracellular target for nitration and enhancement of chaperone expression and ubiquitination. *Biol. Chem.* **381**: 121-126.
185. Hellberg CB, Boggs SE, Lapetina EG (1998). Phosphatidylinositol 3-kinase is a target for protein tyrosine nitration. *Biochem. Biophys. Res. Commun.* **252**: 313-317.
186. Yi D, Smythe GA, Blount BC, Duncan MW (1997). Peroxynitrite-mediated nitration of peptides: characterization of the products by electrospray and combined gas chromatography-mass spectrometry. *Arch. Biochem. Biophys.* **344**: 253-259.
187. Shevchenko A, Wilm M, Vorm O, Mann M (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* **68**: 850-858.
188. Haqqani AS, Cowling RT, Maroun JA, Birnboim HC (1999). Characterization of a polyclonal antibody to human thymidylate synthase suitable for the study of colorectal cancer specimens. *J. Histochem. Cytochem.* **47**: 1563-1574.
189. Souza JM, Daikhin E, Yudkoff M, Raman CS, Ischiropoulos H (1999). Factors determining the selectivity of protein tyrosine nitration. *Arch. Biochem. Biophys.* **371**: 169-178.
190. Harp JM, Hanson BL, Timm DE, Bunick GJ (2000). Asymmetries in the nucleosome core particle at 2.5 Å resolution. *Acta Crystallogr. D. Biol. Crystallogr.* **56 Pt 12**: 1513-1534.
191. Souza JM, Choi I, Chen Q, Weisse M, Daikhin E, Yudkoff M, Obin M, Ara J, Horwitz J, Ischiropoulos H (2000). Proteolytic degradation of tyrosine nitrated proteins. *Arch. Biochem. Biophys.* **380**: 360-366.
192. Sarver A, Scheffler NK, Shetlar MD, Gibson BW (2001). Analysis of peptides and proteins containing nitrotyrosine by matrix-assisted laser desorption/ionization mass spectrometry. *J. Am. Soc. Mass Spectrom.* **12**: 439-448.
193. Beckman JS, Koppenol WH (1996). Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and the ugly. *Am. J. Physiol. Cell Physiol.* **271**: C1424-C1437.

194. Ischiropoulos H (2002). Introduction to serial reviews: reactive nitrogen species, tyrosine nitration and cell signaling. *Free Radic. Biol. Med.* **33**: 727.
195. Balazy M, Kaminski PM, Mao K, Tan J, Wolin MS (1998). S-Nitroglutathione, a product of the reaction between peroxynitrite and glutathione that generates nitric oxide. *J. Biol. Chem.* **273**: 32009-32015.
196. Feelisch M, Stamler JS (1996). Donors of nitrogen oxides. *In: Methods in nitric oxide research*. Editors: Feelisch M, Stamler JS. New York: John Wiley & Sons, Inc. pp. 71-115.
197. Lipton SA (2001). Physiology. Nitric oxide and respiration. *Nature* **413**: 118-9, 121.
198. Fantone JC, Duque RE, Davis BH, Phan SH (1989). 3-Deaza-adenosine inhibition of stimulus-response coupling in human polymorphonuclear leukocytes. *J. Leukocyte Biol.* **45**: 121-128.
199. Mallis RJ, Thomas JA (2000). Effect of S-nitrosothiols on cellular glutathione and reactive protein sulfhydryls. *Arch Biochem Biophys* **383**: 60-69.
200. Jones JJ, McGilvray ID, Nathens AB, Bitar R, Rotstein OD (1997). Glutathione depletion prevents lipopolysaccharide-induced local skin inflammation. *Arch. Surg.* **132**: 1165-1169.
201. Zai A, Rudd MA, Scribner AW, Loscalzo J (1999). Cell-surface protein disulfide isomerase catalyzes transnitrosation and regulates intracellular transfer of nitric oxide. *J. Clin. Invest* **103**: 393-399.
202. Jaiswal M, LaRusso NF, Nishioka N, Nakabeppu Y, Gores GJ (2001). Human Ogg1, a protein involved in the repair of 8-oxoguanine, is inhibited by nitric oxide. *Cancer Res.* **61**: 6388-6393.
203. Broquist HP (1992). Buthionine sulfoximine, an experimental tool to induce glutathione deficiency: elucidation of glutathione and ascorbate in their role as antioxidants. *Nutr. Rev.* **50**: 110-111.
204. Whitcomb JM, Schwartz AG (1985). Dehydroepiandrosterone and 16 alpha-Br-epiandrosterone inhibit 12-O- tetradecanoylphorbol-13-acetate stimulation of superoxide radical production by human polymorphonuclear leukocytes. *Carcinogenesis* **6**: 333-335.
205. Nagano N, Ota M, Nishikawa K (1999). Strong hydrophobic nature of cysteine residues in proteins. *FEBS Lett.* **458**: 69-71.
206. Hur MW, Edenberg HJ (1995). Cell-specific function of cis-acting elements in the regulation of human alcohol dehydrogenase 5 gene expression and effect of the 5'-nontranslated region. *J. Biol. Chem.* **270**: 9002-9009.

207. Keller DA, Heck HD, Randall HW, Morgan KT (1990). Histochemical localization of formaldehyde dehydrogenase in the rat. *Toxicol. Appl. Pharmacol.* **106**: 311-326.
208. Hess DT, Matsumoto A, Nudelman R, Stamler JS (2001). S-nitrosylation: spectrum and specificity. *Nat. Cell Biol.* **3**: E46-E49.
209. Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.
210. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (2002). Analysis of proteins: Immunoblotting and Immunodetection. *In: Current protocols in molecular biology* vol. 2. Editors: Albright LM, Coen DM, Varki A. Massachusetts: John Wiley & Sons, Inc. p. 10.8.1-10.8.17.