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THE ONCOGENIC TRANSFORMATION OF T51B RAT LIVER EPITHELIAL CELLS ALTERS THEIR SENSITIVITY TO APOPTOTIC STIMULI

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Abstract

Rat liver non-parenchymal epithelial cells were stably transfected with the following plasmids: i) pRSVNeo containing a neomycin resistance gene (Neo) under control of the Rous sarcoma virus (RSV) long terminal repeat element and ii) pMT3 containing the Neo gene and the coding sequence of the polyoma middle T antigen (mT) under control of the SV40 enhancer region. Soft agar assays revealed no neoplastic transformation of cells expressing pRSVNeo plasmids, however overexpression of pMT3 plasmids generated a highly tumorigenic cell line. Two approaches were used to induce apoptosis in these cell lines, i.e. serum withdrawal and treatment with a chemotherapeutic drug, teniposide (VM26). The Neo cells were resistant to serum deprivation and sensitive to the VM26 treatment. In contrast, the highly transformed mT cells were very sensitive to the lack of growth factors but became extremely resistant to the VM26 treatment. These cells could arrest their growth and survive for 2 days in the presence of 10 μM VM26. Under the same experimental conditions, the viability of Neo cells dropped and they could only survive for a few hours. Analysis of several growth arrest and DNA-repair related proteins was performed and correlated with the cells' responsiveness to apoptosis. For example, in the VM26-resistant mT cells, the level of DNA repair-associated proteins was upregulated in response to the drug treatment. The results of this study clearly demonstrated that the mT protein brought about cellular phenotypic and metabolic changes, manifested not only as tumorigenic transformation, but also as a resistance to treatment with the chemotherapeutic drug, VM26.

To my family

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Abbreviations

ATP	adenosine 5'-trisphosphate
Bax	Bcl-2-associated protein x
Bcl-2/ Bcl-x	B-cell lymphoma-2/ B-cell lymphoma-protein x
bp	base pair(s)
Cdk	cyclin-dependent kinase
cpm	counts per minute
CY3	indocarbocyanine
DAG	diacylglycerol
dATP	deoxyadenosine 5'-trisphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanosine 5'-trisphosphate
(c)DNA	(complementary) deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNA pol	DNA polymerase
DSB	double strand breaks
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethylene glycol-bis N,N,N',N'-tetra-acetic acid
ERK1/ ERK2	extracellular-regulated protein kinases 1 and 2
g	gravitational force
GADD45	growth arrest DNA damage inducible transcript
h	hour(s)
HMW	high molecular weight

kbp	kilobase pairs
kDa	kilodalton
MAPK(K)(K)	mitogen-activated protein kinase (kinase) (kinase)
MDR	multidrug resistance
min	minute(s)
MTT	(3, (4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide)
NADH	nicotinamide adenine dinucleotide
NP40	nonidet P-40
PAb	polyclonal antibody
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PFGE	pulsed field gel electrophoresis
PIK	phosphatidyl inositol triphosphate
PKC	protein kinase C
PP2A	protein phosphatase 2A
(m)RNA	(messenger) ribonucleic acid
SDS	sodium dodecyl sulphate
Thr	threonine
Tyr	tyrosine
UV	ultraviolet
VM26	teniposide
w/w	weight per weight
w/v	weight per volume

Introduction

I. The Biology of Cancer

1.1 Characteristics of Carcinogenesis

A major feature of all higher eukaryotes is the defined life span of the organism, a property that extends to the individual somatic cells. The majority of cells in a multicellular organism are differentiated. During the process of differentiation, cells develop a specific morphology and function, and lose the ability to proliferate. In those that are not differentiated, growth and division are highly regulated by the processes of mitosis and programmed cell death. Cancer cells are an exception to this extensively controlled system in multicellular organisms. Cancer is a disease that reflects disturbances of the most fundamental rules of behavior in eukaryotic cells. Tumorigenic cells proliferate regardless of whether or not there is a requirement for new cells, and their differentiation is impaired. In fact, these cells are defined by two heritable properties; they and their progeny reproduce in defiance of normal constraints, and they invade and colonize territories normally reserved for other cells (Alberts et al. 1989). Metastasis describes a feature by which the cancerous cell gains the ability to invade normal tissue and establish a new colony. This process marks the distinction between a tumour that is clinically benign and one that is malignant. The ability of cancer cells to metastasize to other tissues and generate more tumors is the major property that leads to death of their host (Tannock and Hill 1997). In general, mutations in the genome cause cancer. Evidence for the genetic origin of this disease includes observations that most carcinogens are also mutagens and that defects in DNA repair are

likely to result in the formation of cancer. Chemical carcinogens comprise numerous compounds that have similar structural features and a common mode of action. They are able to adopt reactive electrophillic forms which can bind DNA in a covalent, but non-specific manner. If DNA repair is prevented, the resulting damage becomes heritable. Ionizing and ultraviolet (UV) radiation, in the form of solar rays and diagnostic or therapeutic treatments can all cause heritable molecular damage and hence, cancer. Heritable forms of cancer also result from chromosome instability initiated in germ cells. This is the case in many cancers including retinoblastoma, chronic myelogenous leukemia and Burkitt's lymphoma (Tannock and Hill 1987).

The genetic mutations that initiate neoplastic events result in the disruption of normal restraints on cell proliferation. Since some parts of the cell machinery that are used to regulate proliferation are nearly identical in many cell types, these cells are likely to be similarly vulnerable. In fact, mutations in a relatively small set of regulatory genes are responsible for much of the loss of cell division control in cancer. These regulatory genes usually take one of two forms. They either stimulate proliferation, and are referred to as **proto-oncogenes**, or they prevent cell division and are called **tumor suppressor genes**. Cancer-causing mutations in these genes result in either the overactivity of a stimulatory gene to create an **oncogene**, or the inhibition of a tumor suppressor gene. In addition to these mutations, there is another type of genetic change caused by viruses that can lead to cancer. They can introduce their DNA into the cell and subvert the cell division control system, (Alberts et al. 1989, Levine 1991).

1.2 Oncogenes

The critical events during neoplastic transformation are the mechanisms which convert proto-oncogenes to oncogenes, a process known as "oncogene activation". There are numerous forms of genetic disruption that can convert a proto-oncogene into an oncogene. Since mutations in a proto-oncogene are dominant-acting, only one allele needs to be affected. The allele may be altered by a point mutation in a protein coding region causing the production of a hyperactive protein, or by a mutation in an adjacent control region, causing overexpression of a normal protein. Gene amplification may also initiate oncogene activation, as is frequently the case with *c-myc*. Furthermore, chromosomal rearrangements, such as a translocation event or fusion of a highly expressed gene with one that is transcribed less often, may result in the overproduction of a normal protein. Burkitt's lymphoma and chronic myelogenous leukemia are both a result of translocations involving, respectively, the *c-myc* and *c-abl* genes (Glover and Hames 1989).

Control of cell proliferation is normally exerted by growth factors. Growth factors, such as epidermal growth factor (EGF), insulin and insulin-like growth factor (IGF), and platelet-derived growth factor (PDGF) act via specific cell surface receptors. These receptors are activated by ligand binding. Their stimulation generates second messengers and a signal transduction pathway ultimately resulting in DNA synthesis (Glover and Hames 1989). The functions of many proto-oncogene products can be fitted into these signaling pathways (Studzinski 1989). While some oncoproteins can directly mimic growth factors, some such as c-src are functionally similar to the tyrosine kinase activity seen on the cytoplasmic domains of activated transmembrane growth factor receptors. In fact, many protein kinases

encoded by proto-oncogenes are associated with cell membranes whether or not they serve a receptor function (Studzinski 1989).

1.3 Tumor Viruses

Carcinogenesis can also occur as a result of viral infection. In fact, 20% of human cancers have a viral association. For example, human papilloma virus (HPV) is linked to genital epithelial carcinoma, Epstein-Barr virus (EBV) is associated with Burkitt's lymphoma, hepatitis B virus (HBV) is strongly implicated in liver carcinoma and human T-cell leukemia virus type I (HTLV-I) is a major risk factor for adult T-cell leukemia.

There exist both RNA and DNA tumor viruses. RNA tumor viruses are known as retroviruses. Retrovirus-infected host cells release progeny viruses from the cell surface by budding. The virus-encoded reverse transcriptase creates a double-stranded copy of DNA from the single strand of viral RNA. This DNA is incorporated into the host cell genome, and is replicated as part of the host genetic material. Cell transformation occurs as a result of the virus-stimulated increase in proliferation. Rous sarcoma virus is the best known retrovirus.

DNA tumor viruses enter the cell and cause neoplastic transformation by one of two methods. Either the genetic material of the virus can integrate into the host cell genome or it can form a plasmid that replicates in a controlled manner without killing the cell. The presence of this viral DNA results in a genetic change that causes the host cell to proliferate in an uncontrolled manner. In some cases, viral proteins in infected cells associate with cellular proteins. This property is demonstrated by the extensively studied DNA tumor

viruses, the Papova viruses Simian virus 40 (SV40) and polyoma virus. SV40 has been shown to associate with the tumor suppressor proteins p53 and retinoblastoma (Rb). Middle T antigen of the polyoma virus binds directly to the proto-oncogene c-src (Barbanti-Brodano et al. 1995).

The murine Polyoma virus belongs to the family of Papova viruses, which are small double-stranded circular DNA viruses. This virus was first discovered by Ludwig Gross in 1953 and was subsequently found to cause a wide variety of tumors in mice. Polyoma virus comprises three T antigen proteins (small T [sT], middle T [mT] and large T [lT]) and three viral capsid proteins. Although they share a 79 amino acid sequence at their amino terminal, the three T antigen proteins are characterized by distinct cellular localization, function and tumorigenic ability (Treisman et al. 1981). Although the precise cellular location and function of sT remains unknown, it binds protein phosphatase 2A (PP2A) and it is believed to play a role in viral replication. The lT antigen is a nuclear protein which acts as a transcription factor in addition to inducing immortalization of polyoma virus-infected cells. Among the three antigens, the middle T antigen has been recognized to be the protein responsible for cell transformation. The middle T antigen is a 55kDa cytoplasmic phosphoprotein which associates with plasma membranes. It does not have any functionality by itself, but gains its oncogenic activity by binding to host proteins and altering their specific function (Dilworth 1995). Specifically, it is the carboxy terminus of mT that is required for attachment to cell membranes, associated protein kinase activities and cell transformation (Carmichael et al. 1982).

1.4 Tumor Suppressor Genes

In addition to the activation of oncogenes by genetic mutation or viral infection, carcinogenesis may involve the loss of tumor suppressor genes. These genes normally exert negative control on cell proliferation. The negative control is expressed as a signal to the cell to decrease its rate of growth and increase its level of differentiation. Mutations that contribute to cancer include deletion or inactivation of a tumor suppressor gene. These cancers are recessive, since they only occur if both copies of the gene are deleted. Tumor suppressor gene deletion has been associated with Wilms' tumor of the kidney, ductal breast carcinoma, hepatoblastoma and retinoblastoma.

One example of a tumor suppressor gene is the Retinoblastoma (*Rb*) gene. The Rb protein is a 110kDa phosphoprotein that is located in the nucleus. Although the amount of Rb in the cell does not change, its activity is regulated by phosphorylation events. Highly phosphorylated Rb has been found at the G1/S boundary and through G2 and S phases of the cell cycle. Lightly phosphoryated Rb has been found in resting cells, and it is this form of the protein that is thought to inhibit cell cycle progression. Transforming proteins of several DNA tumor viruses, such as E1 of adenovirus, SV40 large tumor antigen and the E7 protein of human papillomavirus, were previously found to bind to Rb protein. It has been suggested that these oncogenic viruses sequester the Rb tumor suppressor gene and thereby impair its function (Vile 1992, Alberts et al. 1989).

The most significant member of the tumor suppressor family is the protein p53. p53 is the most commonly mutated gene in human tumors noted to date (Culotta and Koshland 1993). p53, sometimes known as "the guardian of the genome", acts to coordinate a complex

system of responses to DNA damage. This protein regulates the progression of the cell through the cell cycle and is implicated as a checkpoint protein at the G1/S phase transition (Levine 1997). Double-strand DNA breaks are the initial signal that result in p53 stabilization. When DNA damage is detected, wild type p53 may cause growth arrest in G1 or it may send the cell into a pathway leading to apoptosis (Culotta and Koshland 1993). P53 activates the transcription of a variety of genes that lead to G1/S cell cycle arrest. The tumor suppressor may prevent transcription of genes whose products are required for cell cycle progression, or it may stimulate target genes necessary for cell cycle arrest (Cox and Lane 1995). In addition, p53 can activate genes required to initiate the apoptotic pathway. Three principal protein products involved in p53-mediated events are $p21^{waf\text{-}1}$, GADD45 and Bax.

II: Genome Surveillance

2.1 p53 protein

The p53 protein functions primarily as a transcription factor. It enhances the rate of transcription of 6-7 known genes which in turn carry out the p53-dependent functions in a cell. Human p53 consists of 393 amino acids and is divided structurally and functionally into 4 domains. Most importantly, the N-terminal domain, which comprises 42 amino acids, is the transcriptional activation domain that interacts with the cellular transcription machinery to positively regulate gene expression. Specifically, it is the amino acids F19, L22 and W23 that are required for transcriptional activation (Levine 1997, Lane et al. 1994). The sequence-specific DNA binding domain of p53 is localized between amino acids 102

and 292. The majority of p53 mutations, most commonly missense mutations, occur in this region. In fact, more than 90% of missense mutations in p53 occur in the DNA binding domain, 40% of these in residues R175, G245, R248, R249, R273 and R282. These amino acids play a role in either the structural integrity of this domain or in the DNA contact sites. Residues R248 and R273 are the two most frequently altered amino acids in the protein. Mutations in either of these result in defective contacts with DNA and loss of p53 transcription factor activity. There are also mutations which disrupt the structural basis of the β sheet and loop-sheet-helix motif, by altering the conformation of the protein, and obliterating its function as a tumor suppressor (Levine 1997).

P53 response is typically triggered by stress factors. It was originally discovered that levels of this protein rose dramatically in cultured fibroblasts exposed to UV light. Several different types of DNA damage can activate p53, including double-stranded breaks produced by γ-irradiation, UV radiation or topoisomerase-targeting drugs and chemical damage to DNA (Götz and Montenarh 1995). Although the activation of the protein following DNA damage is referred to as an "induction", *de novo* transcription of p53 is not required to produce the high protein levels detected. Normally, the amount of p53 in cells is so small as to be virtually undetectable. This is reinforced by its very short half-life which is controlled by the ubiquitin-mediated protein degradation pathway (Cox and Lane 1995). The major mechanism for p53 accumulation after DNA damage is an increase in the stability of the protein. However, the molecular basis for this stability is unclear (Cox and Lane 1995, Kastan et al. 1991, Kuerbitz et al. 1992, Lu et al. 1992, Fritsche et al. 1993, Lu and Lane 1993). The extent of p53 stabilization is proportional to the amount of DNA damage (Levine

1997). The presence of DNA double strand breaks appears to be sufficient for protein stabilization. It has yet to be determined if p53 itself detects DNA damage and then causes its own upregulation, or if there is a damage-detecting protein which recognizes DNA breaks and "reports back" to p53 (Levine 1997).

2.2 $p21^{waf-1}$

p21 has been isolated independently as a wild-type p53 activated fragment-1 (Waf-1, El-Diery et al. 1993), as a Cdk-interacting protein-1 (Cip-1, Harper et al. 1993), as a senescent cell-derived inhibitor-1 which blocks cell entry into S phase (Sdi-1, Noda et al. 1994), and as a melanoma differentiation associated gene-6 (Mda-6, Jiang et al. 1994) whose expression is induced as a function of terminal differentiation of melanoma cells. It has been demonstrated that DNA damage causes p53-dependent activation of $p21^{waf-1}$ transcription, (Cox and Lane 1995, Gartel et al. 1996). The 21 kDa protein can induce G1 arrest and block entry into S phase by inactivating Cdks (cyclin-dependent kinases) (Xiong et al. 1993) or by inhibiting activity of proliferating cell nuclear antigen (PCNA). In normal cycling cells, $p21^{waf-1}$ exists as a quaternary complex with cyclin, Cdk and PCNA (Zhang et al. 1993).

Two different domains of the p21 protein are responsible for its activity. The N-terminal domain of $p21^{waf-1}$ contains the Cdk inhibitory region. P21 effectively inhibits Cdk2, Cdk3, Cdk4 and Cdk6, which all have a direct role in the G1/S transition (Gartel et al. 1996). The C-terminal domain of $p21^{waf-1}$ is involved with binding to PCNA. The available evidence suggests that $p21^{waf-1}$-PCNA complexes block the role of PCNA as a DNA polymerase processing factor in DNA replication, but not its role in DNA repair. Thus the

protein prevents replication of damaged DNA. It is believed that $p21^{waf-1}$ serves as a bridge between cyclin-Cdk and PCNA. Thus, $p21^{waf-1}$ can interact with cyclin-CDK complexes, in addition to PCNA to exert its inhibitory effect on the entry of cells into S phase (Levine 1997).

2.3 GADD45

Another component of the p53-dependent growth arrest pathway is GADD45, the 19kDa growth arrest and DNA damage inducible transcript (Smith et al. 1994). GADD45 is a cell cycle regulated nuclear protein which reaches maximal expression levels in the G1 phase of the cell cycle. It is induced following DNA damage by ionizing radiation (IR), UV radiation, chemotherapeutic drugs such as etoposide and other stresses such as hypoxia. When overexpressed, the protein exerts a negative effect on cell growth (Fornace and Smith 1996, Hall et al. 1995, Smith et al. 1994). The increase in GADD45 requires an intact p53-dependent pathway. In fact, a p53-binding site is found on the third intron of the GADD45 gene (Fornace and Smith 1996, Hall et al. 1995). However, in some cases, the elevated GADD45 expression is p53-independent via an unclear mechanism (Kelman 1997). Recently it has been shown that $p21^{waf-1}$ also interacts with GADD45. It is the carboxy terminal of $p21^{waf-1}$ that binds the protein. This region on $p21^{waf-1}$ overlaps the PCNA binding domain. It is believed that GADD45 may regulate the availability of $p21^{waf-1}$ and modulate its ability to interact with PCNA. The amino terminal of GADD45 itself can also bind PCNA, and the two have been shown to associate following DNA damage. However, the mechanism of action of GADD45 on PCNA is unknown (Hall et al.1995, Kelman 1997,

Gartel et al. 1996).

2.4 DNA-Dependent Protein Kinase

DNA double-strand breaks (DSB) are created by a variety of agents such as chemotherapeutic drugs and ionizing radiation and are also formed as intermediates in certain recombination reactions. For instance, the antineoplastic drug VM26 (teniposide) introduces DSB in DNA by interfering with the breakage-resealing activity of topoisomerase II (Roy et al. 1992). DSB are also created between recombination signal sequences and coding gene segments during V(D)J recombination. V(D)J recombination occurs during the development of B and T cells. In this process, the wide variety of alternative immunoglobulin and T-cell receptor genes is created by bringing together variable (V), diversity (D) and joining (J) subexonic gene segments in various recombinations (Jackson and Jeggo 1995).

Misrepaired or unrepaired DNA DSB may result in inactivation of a single gene, translocations or the actual loss of large segments of the genome. All of these events may have significant consequences for the cell, including the inactivation of essential cellular components, leading to cell death, or the inactivation of tumour suppressor genes, leading to cancerous growth (Jackson and Jeggo 1995). Given the serious implications of unrepaired DSB, highly efficient mechanisms have evolved which detect and repair DNA damage. In response to DNA damage, cells transduce a signal resulting in stabilization of p53 protein and subsequent activation of one of two pathways, either apoptosis or cell cycle arrest.

Although many DNA damage-response genes are known, the molecule that detects

DNA damage and initiates the p53 response has not been identified. One candidate molecule is DNA-dependent protein kinase (DNA-PK), a serine/threonine protein kinase that is activated by DNA DSB (Rathmell et al. 1997). Initial work with DNA-PK revealed its ability to phosphorylate DNA-binding transcription factors (Jackson and Jeggo 1995). Although DNA-PK is not required for the accumulation of p53 and cell cycle arrest after DNA damage, it has been shown to phosphorylate p53, in addition to c-myc, c-fos, c-jun and topoisomerases I and II (Anderson 1993).

DNA-dependent protein kinase (DNA-PK) was discovered as an activity in cell extracts that caused the DNA-dependent phosphorylation of various endogenous proteins (Jackson 1997). The kinase is required for repairing DNA double-strand breaks and for site-specific V(D)J recombination. It comprises a catalytic subunit, DNA-PK_{CS}, and a regulatory subunit, Ku. Ku is a heterodimer of approximately 70kDa and 80kDa (Ku70 and Ku80). It was originally identified as a human autoantigen associated with lupus erythematosus, scleroderma overlap syndrome and rheumatoid arthritis (Lees-Miller 1996). The DNA-PK_{CS} is a 460kDa polypeptide that is normally inactive and has the unusual property of being active only when bound to DNA (Jackson and Jeggo 1995). It is capable of binding without specificity to DNA ends, but it gains its activity only when it binds DNA at DSB. It is not activated by DNA nicks and hairpin ends. Although DNA-PK is capable of acting as a self-contained kinase that is activated by binding directly to DNA, Ku plays a role in the stabilization of its binding to DNA ends (Hammarsten and Chu 1998).

Ku is a fairly ubiquitous nuclear protein that binds with high affinity to the ends of DNA and is capable of ATP-dependent translocation along DNA (Lees-Miller 1996). It is

autophosphorylated in vitro, but lacks an identifiable DNA-PK phosphorylation site. Autophosphorylation of Ku does not appear to affect DNA-PK binding (Jeggo 1997). It is thought that Ku may protect DNA ends from exonucleolytic degradation (Anderson and Carter 1996). It has been reported to be associated with DNA replication complexes and recombinant Ku has been shown to have ATP-dependent helicase and DNA-dependent ATPase activities (Tuteja et al. 1994).

The model for DNA-PK assembly on a DNA end begins with high affinity binding of Ku to DNA ends. It translocates to an internal position, uncovering the DNA ends and forming a more stable complex with the DNA. The uncovered DNA end is then bound by p460 to form the DNA-PK complex. Cooperative interaction between p460, Ku and the DNA lead to activation of the kinase (Lees-Miller 1996, Hammarsten and Chu 1998).

Independently, neither Ku nor DNA-PK has significant kinase activity. Co-immunoprecipitation studies suggest that Ku and DNA-PK_{CS} interact only in the presence of DNA (Lees-Miller 1996). Cells defective in DNA-PK_{CS} or Ku are deficient in DNA DSB repair and, consequently, are hypersensitive to ionizing radiation. Cells deficient in Ku have shortened telomeric chromosomal ends (Jackson 1997).

III. Apoptosis

3.1 Apoptotic Features

Apoptosis, a naturally-occurring form of cell suicide, is an often gene-directed physiological pathway important for tissue development and homeostasis. It is a fundamental feature of multicellular organisms which contributes to the elimination of many

cells during organogenesis.

Historically, the first form of cell death to be recognized was necrosis. Necrotic death results from stimuli such as severe hypoxia, hypothermia, lytic viral infection or exposure to a variety of toxins and respiratory poisons. These diverse lethal stimuli increase the permeability of the plasma membrane, either through alterations in its structure or by failure of the cationic membrane pumps. Under these circumstances, Na+, K+, Ca++ and Mg++ move down concentration gradients, and the cell becomes unable to regulate osmotic pressure. At first, a series of reversible changes occur. The endoplasmic reticulum dilates, the cell begins to swell and its membrane changes shape, its mitochondria become denser and protein synthesis declines. This period is followed by a series of irreversible changes. The mitochondria swell, and the cell itself swells, leading to a lethal disruption of cellular membranes, causing the cell to burst (Wyllie and Duvall 1986, Cohen 1993). Necrosis involves traumatic injury to tissues. Cells release their protein and nucleic acid contents, which results in severe inflammatory responses. This type of death is associated with major perturbations in the cellular environment and has been regarded as a pathological response.

In contrast, apoptosis has been observed in many instances where the death of the cell is not pathological, but appears to be part of homeostatic regulation (Grindley 1994). Apoptosis differs from necrosis in a number of ways. A cell undergoing apoptosis will become rounded as it severs its junctions with neighboring cells. At the same time, the cytoplasm condenses but the morphology of mitochondria and ribosomes are preserved. The endoplasmic reticulum dilates and forms vesicles which tend to fuse with the surface membrane, giving a characteristic "blebbing" appearance. The chromatin rapidly forms

dense crescent-shaped aggregates lining the nuclear membrane, and the nucleolus fragments. Fragmentation of DNA occurs and often a characteristic ladder pattern, representative of oligonucleosomal DNA, can be seen on electrophoresis gels (Wyllie and Duval 1986, Grindley 1994). In addition, complex invaginations develop in the nuclear membrane, segmenting the nucleus. The plasma membrane also becomes convoluted so that the cell separates into a cluster of membrane-bound fragments, "apoptotic bodies", which often contain morphologically normal mitochondria and other organelles (Wyllie and Duval 1986). Finally, the cell displays new antigens which signal to immune system cells that it is ready for phagocytosis (Grindley 1994). Cells in this state are rapidly recognized, phagocytosed and digested either by macrophages or epithelial cells. This removal of apoptotic cells occurs so quickly that examination of normal tissues rarely reveals any cells in the process of apoptosis (Grindley 1994).

Apoptosis is observed when elimination of a cell is part of organized tissue reactions. For example, during embryogenesis in the disappearance of interdigital cells, during the formation of the digits from the solid limb paddle, during metamorphosis in resorption of the tadpole tail, during endocrine-dependent tissue atrophy in the thinning of the adrenal cortex of the rat after birth and during control of normal tissue turnover in post-lactation breast tissue. Apoptotic events are also evident in regressing tumors (Wyllie and Duvall 1986). In addition to controlling developmental events, this physiological pathway is required to remove damaged and potentially dangerous cells from tissues. For example, autoreactive and non-functioning cells of the immune system are removed by apoptosis. Cells with damaged genetic material are also removed. In instances where perturbations in

the apoptotic response prevent removal of damaged or abnormal cells, severe illness is usually the result. For example, defects in this pathway have been implicated in the etiology of cancer, AIDS, autoimmune disease and degenerative diseases of the central nervous system (Carson and Ribeiro 1993, Edgington 1993, Sen and D'Incalci 1992).

A variety of signals can induce apoptosis. Often the signal to die comes from the environment, as in exposure to or withdrawal of a hormone or growth factor. In other cases, as in the case of neutrophil turnover, the cell has an autonomous internal clock controlling its death program (Cohen 1993).

One significant class of therapeutic inducers of apoptosis is that of DNA-damaging drugs such as chemotherapeutic drugs (Grindley 1994). Many anti-cancer drugs stimulate apoptosis in sensitive cells. For example, topoisomerase inhibitors, alkylating agents, anti-metabolites and hormone antagonists are used for chemotherapy. The use of apoptotic stimuli to eliminate neoplastic tissue is extremely beneficial. The dying cells are rapidly phagocytosed by other cells, and provide no risk to the surrounding tissue (Edgington 1993, Carson and Ribeiro 1993).

The process of apoptosis frequently requires the synthesis of new proteins. In fact, protein synthesis is initiated after the apoptotic stimulus, and apoptosis can be blocked if transcription or translation are inhibited (Cohen 1993). Individual proteins have been associated with apoptosis in two ways: either they are expressed in cells undergoing apoptosis, or their modulation affects the process (Cohen 1993). One example of an apoptosis-modulating protein is the proto-oncogene *c-myc*, which may play a part in regulating the choice between proliferation and apoptosis. Fibroblasts which express *c-myc*

do not undergo growth arrest in low serum concentrations as do wild-type fibroblasts, but they readily undergo apoptosis. The tumor suppressor protein p53 is associated with apoptotic responses in a number of cell types. The apoptotic pathway is one downstream cascade stimulated after p53 detection of DNA damage, and in fact it can be said that p53 is a key determinant in the apoptotic response to DNA damage (Cox and Lane 1995).

A number of factors affect the decision of a cell to enter a p53-mediated apoptotic pathway. Under conditions in which the DNA is irreparably damaged, survival factors are limiting, or when an activated oncogene is forcing cells into a replicative cycle, p53-mediated apoptosis prevails. In this way, cells with an unstable genome can be eliminated before they replicate damaged DNA. In addition, cells can be removed when they contain an activated oncogene that commits them to enter the cell cycle, despite environmental signals (i.e. due to limiting growth factors). These events prevent tumorigenic growth and this is the reason why so many cancerous cells contain mutated p53 (Levine 1997). The p53 protein plays a role in triggering apoptosis under several different physiological conditions. For example, thymocytes and stem cells from mice that receive DNA damage will undergo apoptosis, while in p53 null mice these cells do not. However, not all apoptotic events are p53-dependent. For example, immature thymocytes from p53 null mice die by apoptosis when exposed to glucocorticoids or to compounds that trigger T cell receptor pathways (Levine 1997).

P53 may use transcriptional activation or direct protein signaling to initiate apoptosis. One gene that is regulated by p53 and can influence the decision of a cell to commit to apoptosis is *Bax*.

3.2 Bcl-2 Family

The *Bcl-2* gene codes for a 25kDa protein whose expression is deregulated in cases of follicular B-cell lymphoma. This proto-oncogene is the founding member of a large family of proteins comprising both antagonists and agonists which compete through dimerization to regulate apoptosis. They can either repress apoptosis (i.e. Bcl-2, Bcl-x_L, Mcl-1 and A1) or promote apoptosis (i.e. Bax, Bcl-x_S, Bad and Bak) (Knudson and Korsmeyer 1997). The p53 tumor suppressor protein is a direct transcriptional activator of *Bax*. On the other hand, the promoter for the *Bcl-2* gene has a negative response element for p53 (Maxwell et al. 1997, Götz and Montenarh 1995).

Gene knockout experiments have identified the anti-apoptotic functions of Bcl-2 and Bcl-x, and the pro-apoptotic function of Bax. Bcl-2-deficient mice are hypopigmented, with a low concentration of lymphocytes and an excess of nitrogenous compounds in the blood. Bcl-x deficient mice are embryonic lethal, displaying increased apoptosis of both haematopoietic and neuronal cell types. All of these phenotypic characteristics are the result of increased cell death. In contrast, Bax deficient mice demonstrate an excess of lymphocytes, granulosa cells and spermatogonia as well as select neurons that display marked resistance to neurotrophic factor deprivation (Knudson and Korsmeyer 1997).

Bcl-2 members are able to form dimers, often including homodimer and heterodimer pairs. Bax shows homology to Bcl-2, mainly within two highly conserved regions called the Bcl-2 homology 1 and 2 domains (BH1 and BH2). Bax can form homodimers as well as heterodimers with Bcl-2 and Bcl-x. Select mutations in the BH1 and BH2 domains of Bcl-2 and Bcl-x_L lead to the simultaneous loss of Bax binding and of anti-apoptotic activity. It has

been shown that the death agonists and antagonists compete, at least in part, through dimerization and it is the ratio of antagonists to agonists that influences a cell's decision to enter apoptosis (Korsmeyer 1995). Bcl-2 and Bax have been shown to act in the same genetic pathway and it has been shown that when Bcl-2 is in excess, Bcl-2 homodimers predominate and cells are protected from apoptosis. However, when there is excess Bax, homodimers of this pro-apoptotic protein prevail and cells die by apoptosis (Korsmeyer 1995).

Bcl-2, Bcl-x_L and Bax all contain a hydrophobic segment at their C-terminal end that is believed to serve as a membrane anchor. However, the proteins display different subcellular distributions. Bcl-2 is an integral membrane protein of the inner mitochondrial membrane. This localization seems to be important, since functions of the inner mitochondrial membrane such as oxidative phosphorylation and electron transport are possibly involved in survival mechanisms (Götz and Montenarh 1995). In addition to mitochondrial membranes, Bcl-2 has been localized to the nuclear envelope and the endoplasmic reticulum. On the other hand, Bax is found predominantly as a soluble protein in the cytosol. This diffuse localization of Bax does not change with the co-expression of high levels of Bcl-2 or Bcl-x_L (Wolter et al. 1997).

It was found that induction of apoptosis in murine thymocytes by either dexamethasone or γ-irradiation resulted in a shift in the localization of Bax and Bcl-x_L, from soluble to membrane-bound forms. Also, in HL-60 leukemia cells, Bax movement into membranes was observed following staurospaurine-induced apoptosis (Hsu et al. 1997). Results from confocal microscopy performed on Cos-7 kidney epithelial cells and L929

fibroblasts showed that once initiated, this movement of Bax was completed before cellular shrinkage or nuclear condensation. Removal of the C-terminal hydrophobic domain from the Bcl-2 protein has been shown to cause a range of results from complete abrogation of the survival function of this protein to no effect at all. However, removal of the same domain from Bax inhibited redistribution during apoptosis and inhibited its death-promoting activity (Wolter et al. 1997). The dependency of Bax redistribution on the C-terminal hydrophobic domain suggests that a conformational change or other modification might occur in Bax during apoptosis, allowing the previously inaccessible or blocked hydrophobic tail to insert into membranes. While it remains unclear how this may be controlled, it has been suggested that phosphorylation of Bax and Bcl-x_L may result in their translocation to a membrane from the cytosol (Hsu et al. 1997).

Research Objectives

Cancer is one of the most debilitating diseases known. It often causes a lengthy, painful illness and can affect almost any tissue in the body. Cancer accounts for a huge percentage of human deaths every year. In fact, only heart disease is known to kill more people annually (Vile 1992). Thus the motivation for finding effective methods of treatment for this disease can be well understood.

Strategies for drug therapy have included the use of anti-proliferative agents, targeted to interfere with DNA integrity and replication. Most of these cytotoxic drugs have been shown to induce apoptosis in susceptible cells. However, many cancerous tissues continue to be resistant to treatments. The mechanism of coupling of a drug stimulus to the cell death response is not well understood. It is believed that modulation of this coupling can affect the sensitivity of cancer cells to drug treatment (Hickman 1992, Hickman et al. 1994, Bursch et al. 1992).

The objective of my research was to determine whether middle T antigen of polyoma virus (mT) was capable of producing a neoplastic phenotype in rat liver epithelial cells, and to establish whether the presence of mT altered their sensitivity to apoptosis. Furthermore, I wished to identify the cellular events responsible for any observed changes. This knowledge could then be applied to design better cancer treatment modalities. By effectively exploiting this basic knowledge of the phenomenon, cancer cells could be removed by apoptosis and damage to surrounding tissues avoided. However, it is essential to first establish the responses of transformed cells to various inducers of apoptosis and then to specifically map the metabolic pathways involved.

Materials and Methods

Cell Lines and Culture Conditions

T51B rat liver nonparenchymal epithelial cells, originally established by Dr. Swierenga (1978), were used for this study. The cells were transfected either with the pRSVNeo plasmid, containing the aminoglycosyl phosphotransferase gene under control of the Rous sarcoma virus long terminal repeat element and conferring resistance to the neomycin analogue G418 (T51B Neo), or with the pMT3 plasmid containing the neomycin-resistance gene as well as the coding sequence for the middle T antigen of the polyoma virus (T51B mT) as described by Royal et al. (1992). They were grown at 37°C in 8% CO_2 atmosphere in αMEM medium supplemented with 10% fetal bovine serum (FBS) and 250 μg/ml active G418. All tissue culture reagents were purchased from GibcoBRL, Long Island, NY. The cells were a generous gift from Dr. Normand Marceau (Centre de Recherche en Cancérologie de l'Université Laval, l'Hôtel-Dieu de Québec, Québec, Canada).

Growth Kinetics

Cells were seeded at a density of $80x10^4$ cells per 80 cm^2 culture flask containing 18 ml of αMEM medium supplemented with 10% FBS and 250 μg/ml active G418. Cells were counted every day for five days. For counts, the growth medium was removed and detached cells were collected by centrifugation (Beckman model TJ-6) at 134 g for 5 min. Adherent cells were washed with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2), detached with 0.15% trypsin (Sigma-Aldrich Canada, Oakville, ON) in PBS, then collected by centrifugation and pooled with the floating

cells. Cells were counted in the presence of trypan blue (Sigma-Aldrich) using a hemacytometer.

Growth in Soft Agar

3 ml of 0.6% agar (Difco Laboratories, Detroit, MI) in αMEM medium containing 10% FBS and 250 μg/ml G418, were poured into a 60 mm culture plate and allowed to solidify. Approximately $5x10^4$ cells were then mixed with 2 ml of the 0.6% agar solution and added to the dish of solidified agar. All manipulations were performed at 37°C. The dishes were placed in an incubator and cells were fed once a week with 0.5 ml of αMEM, 10% FBS and 250 μg/ml G418. The plates were examined once a week.

Induction of Apoptosis

Apoptosis was induced either by serum deprivation or by a treatment with teniposide VM26 (Bristol-Meyers Squibb, Candiac, PQ). For growth factor withdrawal, 75% confluent cultures, grown in 80 cm^2 culture flasks were placed in serum-free αMEM containing 250 μg/ml G418. For the drug treatment, 10 μM VM26 was added to cells grown in the complete medium. Both treatments were continued for up to 3 days.

Cell Viability Assays

Cell viability was assessed by trypan blue exclusion and MTT (3, (4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide) assays. The MTT assay was performed essentially as described by Hansen et al. (1989). Briefly, 48 h before the

treatments, cells were seeded in 24-well plates at a density of $1x10^5$ cells per well. After the treatments were completed, 500 µl of fresh medium and 125 µl of 5 µg/µl of MTT solution (Sigma-Aldrich) were added to each well. The plates were incubated for 2 h at 37°C and then 500 µl lysis buffer (20% w/v SDS, 2% acetic acid and 2.5% 1N HCl in 50:50 H_2O:dimethyl formamide) was added. The contents of the wells were vigorously mixed and the optical density was measured at dual wavelength 570nm and 690nm using an Easy Reader 400 AT plate reader (SLT-LabInstruments, Austria). The results were expressed as percentage of control readings obtained from untreated cells. The experiments were repeated 3 times.

Pulsed field gel electrophoresis

PFGE was performed as described by Walker et al. (1991). Cells were grown to 75% confluence in 80 cm^2 flasks, then subjected to serum deprivation and VM26 treatments. Floating and adherent cells were collected by centrifugation for 5 min at 134 g (Beckman model TJ-6). Cell pellets (approximately $2x10^7$ cells/time point) were washed with nuclear homogenization buffer (15 mM Tris-HCl pH 7.4, 1 mM EGTA, 2 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 60 mM KCl, 15 mM NaCl) and resuspended in a mixture of 200 µl nuclear homogenization buffer, 200 µl 1.5% low melting point (LMP) agarose (GibcoBRL) and 5 µl 20 mg/ml proteinase K (Sigma-Aldrich). The solution was then transferred to a 1cc syringe, sealed with parafilm and placed at 4 °C until solidified. After solidifying, the plugs were removed from the syringe, then incubated with rotation at 37 °C for 3 h in a tube containing 3 ml digestion buffer (10 mM NaCl, 10 mM Tris-HCl pH 9.5,

25 mM EDTA, 1 mM EGTA), 20 µl of 20 ml/mg proteinase K and 300 µl of 10% laurylsarcosine (Sigma-Aldrich). The plugs were then washed twice in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM EGTA). They were stored in the syringe at 4 °C until use.

Slices of the plugs (equivalent to $1.25x10^6$ cells) were loaded on 0.8% agarose gels in TBE buffer (892 mM Tris-HCl pH 8.0, 890 mM boric acid, 25 mM EDTA). The wells were sealed with LMP agarose and electrophoresis was carried out by field inversion using horizontal gel chambers (200/20 power supply and Pulsewave 760 switcher, Biorad, Mississauga, ON). After staining with 1 µg/ml ethidium bromide in distilled water, the gels were photographed on a transilluminator with UV light using a Polaroid DS-34 camera (Cambridge, MA).

Isolation of RNA

At specific times after the treatments, total cellular RNA was extracted as described by Favaloro et al. (1980). All manipulations were carried out on ice and all solutions were treated with 1% v/v DEPC (diethyl pyrocarbonate). Briefly, all detached and adherent cells were collected, washed in PBS and resuspended in lysis buffer (140 mM NaCl, 10 mM Tris-HCl pH 7.4, 1.5 mM $MgCl_2$, 5% NP-40) for 3 min on ice. Cell debris were removed by centrifugation at maximum speed (16000 g) in Eppendorf centrifuge 5415C for 30 sec. An equal volume of 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.2% SDS was added to the RNA-containing supernatant and the mixture was extracted twice with 25:24:1 v/v/v phenol:choloform:isoamyl alcohol. RNA was precipitated with 1/20th volume 3.0 M

NaCl and 2 volumes of 95% ethanol overnight at -20 °C. The RNA pellet was washed with 70% ethanol, dried for 15 min and resuspended in TE buffer. The quality of total RNA was assessed by electrophoresis on an agarose gel containing 1 μg/ml ethidium bromide (55 V for 3 hours). Messenger RNA (mRNA) was subsequently isolated from each sample using the Promega PolyATract IV system (Promega, Madison, WI). The RNA was quantitated based on OD_{260}.

Northern blotting

Northern blotting was conducted essentially as described by Sambrook et al. (1989). Approximately 400 ηg of each mRNA sample was separated on a 1% agarose gel containing 8% (v/v) formaldehyde (Sigma-Aldrich Canada, Oakville, ON). The separated mRNA was transferred overnight onto a 0.45 μm Nytran nylon membrane (Schleicher and Schuell, Keene, NH) using 10X SSC buffer (1.5 M NaCl, 150 mM Na_3citrate, pH 7.0) then UV cross-linked onto the membrane using the UV Stratalinker 2400 (Stratagene, La Jolla, CA). The blot was hybridized with appropriate ^{32}P-labelled restriction fragments. Hybridization of probes was performed overnight at 42 °C. The hybridization buffer consisted of 0.5 M Na_2HPO_4, 1 mM EDTA, 1% bovine serum albumin (BSA), 5% SDS (Sigma-Aldrich) and 50% formamide (Sigma-Aldrich). The membranes were washed extensively at room temperature with 2X SSC/0.1% SDS, then with 0.2X SSC/0.1% SDS. The final wash was with 0.2X SSC and 0.1% SDS at 65°C. Blots were exposed overnight at -80 °C to Kodak X-OMAT autoradiographic film (Eastman Kodak Co., NY). For re-probing, the blots were

boiled for 2 minutes in distilled water.

Preparation of cDNA Probes

Plasmids containing cDNA were propagated, isolated and restriction digested as described by Sambrook et al. (1989). Approximately 50 ηg of the appropriate restriction fragment was radiolabelled using [α-^{32}P] dATP and [α-^{32}P] dGTP (800 Ci/mmol, NEN DuPont, Boston, MA) and the Amersham Multiprime DNA labeling system (Amersham, UK). DNA probes were routinely labeled to a specific activity of 1-2 x 10^9 cpm/μg DNA. The human α-tubulin cDNA clone was a generous gift from Dr. N. Cowan (New York University, NY). Human p53 cDNA was purchased from American Type Culture Collection (ATCC, Rockville, MD). Hamster GADD45 cDNA was a generous gift from Dr. A. J. Fornace (National Cancer Institute, National Institutes of Health, Bethesda, MD). Mouse Bax cDNA was a generous gift from Dr. S. Korsmeyer (Howard Hughes Medical Institute, Washington University School of Medicine, Saint Louis, MO).

Immunofluorescent Staining and Microscopy

Cells were grown on glass coverslips, rinsed twice in PBS, then fixed and permeabilized for 10 min in 3.7% formaldehyde, 0.25% gluteraldehyde and 0.5% Triton X-100 in PEM buffer. Free aldehyde groups were reduced with 0.1% sodium borohydride, cells were washed with PBS, and blocked for 30 min in 5% normal serum. The cover slips were incubated with the 1° antibody for 1 hour, washed with PBS, then incubated with the 2° antibody for 45 min in a dark, humidified chamber. Hoechst 33258 (Sigma-Aldrich) was

used to counter stain DNA. 1 μg/ml Hoechst 33258 was added to the cells for 1 min before the two final washings with PBS. Coverslips were mounted onto glass slides using Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA). Stained cells were analyzed under an Olympus BX50 Microscope equipped with phase and epifluourescence optics and photographed using Kodak EPH P1600 slide film.

The following primary antibodies were used: polyclonal anti-ACTIVE™ MAPK (Promega Corporation, Madison, WI) at a dilution of 1:200, polyclonal anti-GADD45 (H-165, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:75 dilution, polyclonal anti-$p21^{waf-1}$ (Santa Cruz Biotechnology) at a dilution of 1:200, polyclonal anti-DNA-PK1 (Dr. Lees-Miller, University of Calgary) at a dilution 1:100, monoclonal rat anti-mT (PAb 815, Dr. Bolen, National Cancer Institute, Bethesda, MD) at a 1:5 dilution. The secondary antibodies, CY3-linked donkey anti-rabbit and goat anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at a dilution of 1:600 and 1:300, respectively.

Data Analysis

Jandel Sigmaplot 3.0 software was used to generate curves and standard error from the growth kinetics curves and cell viability data. The percentage of live and dead cells were calculated from cell counts, and the percent mitochondrial activity was calculated from the MTT assay. Samples were counted in triplicate, and the experiments were repeated 3 to 4 times.

Northern blotting results were corrected for RNA loading. The Macintosh-based program, NIH Image, was used to calculate the densities of the signals from the

autoradiography film.

Slides were scanned using a Nikon IS-1000 scanner to generate .tif files. Images were assembled into multi-image plates using Adobe PhotoShop 4.0.

Results

I. Characterization of experimental cell system

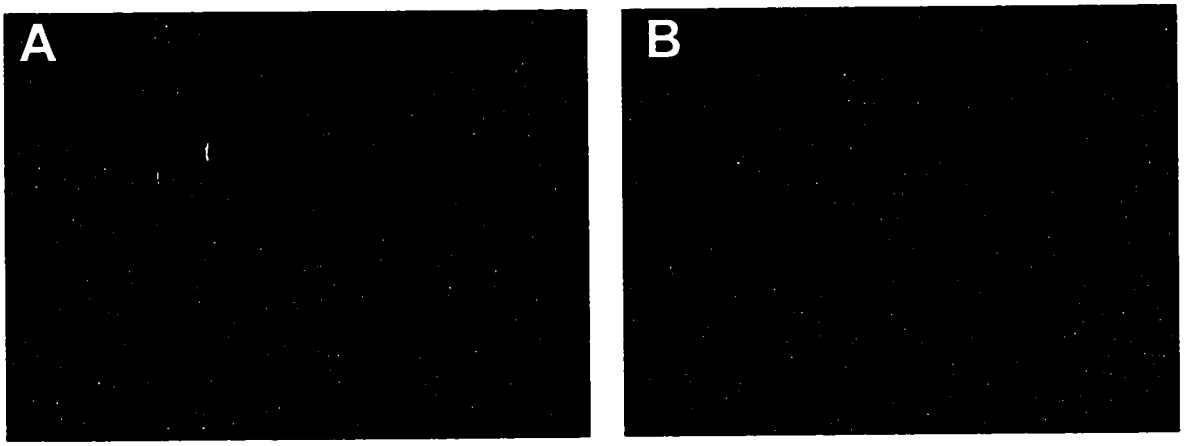
1.1 Expression of middle T antigen

The expression of mT was confirmed by immunofluorescence staining with a monoclonal antibody specific for middle T antigen (Bolen and Israel, 1985). The protein was expressed in the mT cells where it was localized in the cell cytoplasm, predominantly in perinuclear regions, but not in the cell nuclei (Fig. 1A and 1B). No staining was evident in Neo cells.

Middle T antigen has been shown to associate with and inhibit the activity of protein phosphatase 2A (PP2A). As a result, an accumulation of activated MAPK in cells expressing mT was postulated (Dilworth, 1995). In order to confirm that in transfected mT cells this antigen exhibited the same function, we tested the cells for the presence of phosphorylated, and activated p44/p42 MAPKs (aMAPK). For the purpose of detection, an antibody specific for the dually phosphorylated (Tyr^{183} and Thr^{185}) aMAPK was used. Both cell lines were analyzed by immunostaining (Fig. 2). In Neo cells, the aMAPK could be detected exclusively in mitotic cells where the staining was concentrated near chromatin (Fig. 2A and 2B). In contrast, in the mT cells, aMAPK was present in the entire cell population. A strong diffuse fluorescence signal was detected in the cytosol, as well as in the nuclei where it seemed to be associated with granular structures (Fig. 2C and 2D).

Figure 1: Expression of middle T antigen

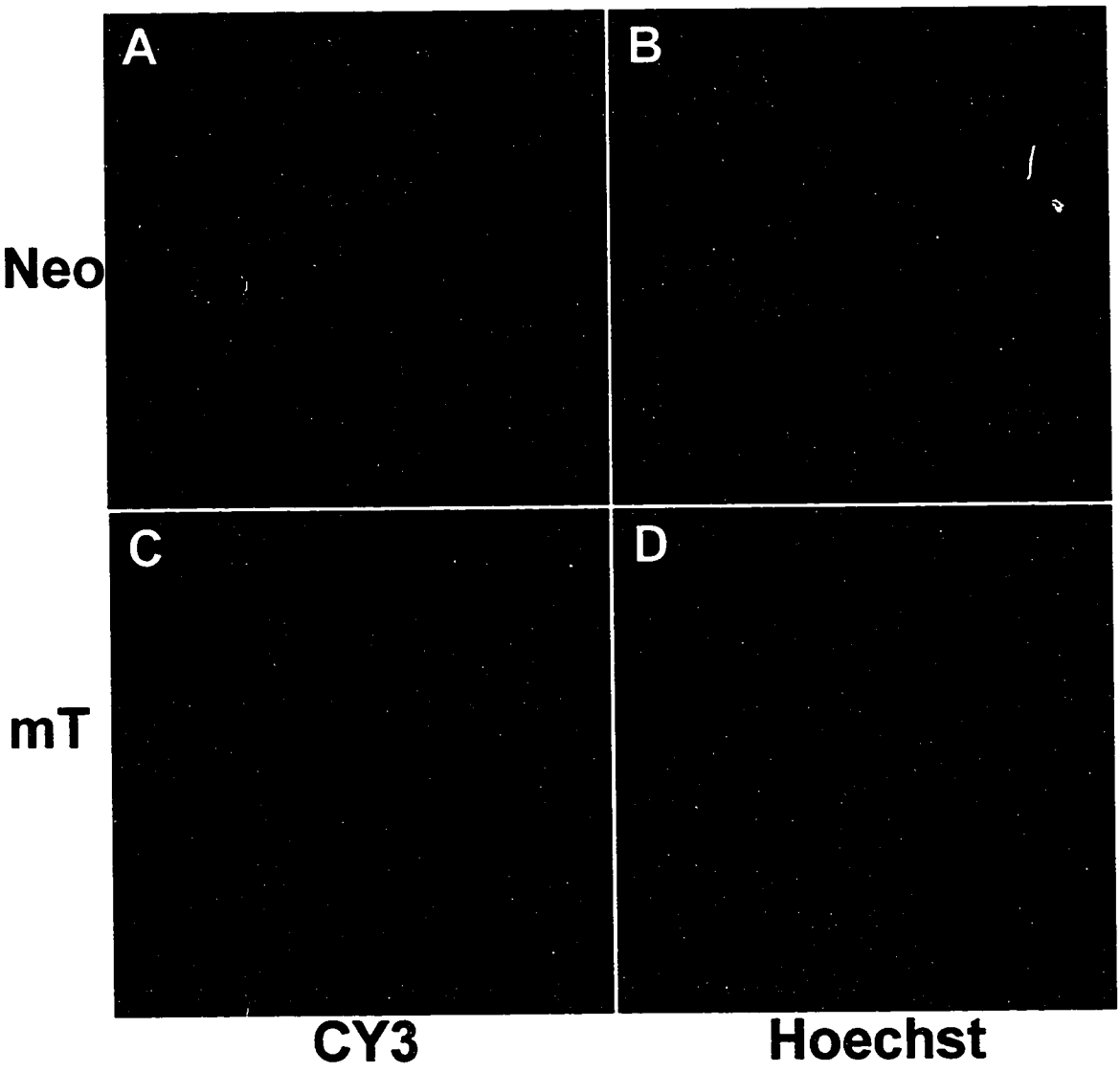
Cells grown on glass cover slips were immunostained with anti-mT antigen mouse monoclonal antibody and CY3-conjugated goat anti-mouse secondary antibody (A). The nuclei were counterstained with Hoechst 33258 dye (B) and the cells were observed under an Olympus BX50 microscope equipped with phase and epifluorescence optics and photographed using Kodak Ektachrome film.
Magnification: 1000X



CY3 Hoechst

Figure 2: Detection of activated MAPK

Cells grown on glass cover slips were immunostained with anti-phosphorylated MAPK rabbit polyclonal antibody and CY3-conjugated donkey anti-rabbit secondary antibody (A, C). The nuclei were counterstained with Hoechst 33258 dye (B, D) and the cells were observed under an Olympus BX50 microscope equipped with phase and epifluorescence optics and photographed using Kodak Ektachrome film.
Magnification: 940X


A
B
Neo
C
D
mT
CY3
Hoechst

1.2 Growth Kinetics

To determine the effect of middle T antigen on the rate of growth of T51B cells, cells were seeded at a density of 80 x 10^4 cells per 80 cm^2 culture flask and were counted in the presence of trypan blue, every day for 5 days after seeding (Fig. 3). No fresh media or sera were added to the flasks for the duration of the experiment. There was no increase in the number of Neo cells within the first 24 hours after seeding, however, after two days, the number of live cells increased 6-fold (Fig. 3A). By the third day after seeding, the population of live cells in the flask had reached 14 million and the cultures were confluent. There was no significant increase in the number of live cells in the subsequent days (i.e. 4, 5) of the experiment. The population of trypan blue-positive dead cells reached a maximum of 20% on the third day following seeding, and remained at that level up to day five.

By contrast, the mT cells started dividing almost immediately after seeding (Fig. 3B). Their number increased 3-fold within the first day. These cells continued to proliferate, and reached a maximum of 31 million cells on day four of the experiment. However, between the fourth and fifth day after seeding, the number of live cells in the flask dropped to 21 million. The population of trypan blue-positive dead cells increased from 20% on the third day to approximately 50% on the fifth day.

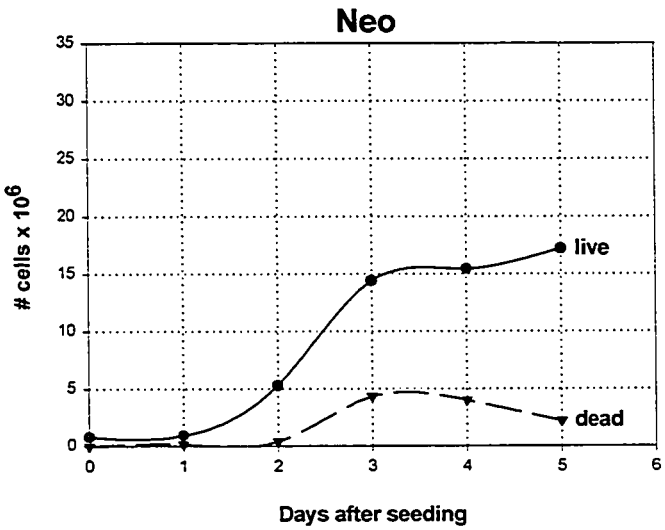
In general, compared to the parental cells, the mT cells had a higher rate of growth, doubling approximately every 18 h, whereas the Neo cells doubled every 24 h. While Neo cells were able to become quiescent upon reaching confluence, this was not the case with the mT cells. They continued to divide and were able to grow to a much higher density, however, their rate of death was also much higher.

Figure 3: Growth kinetics

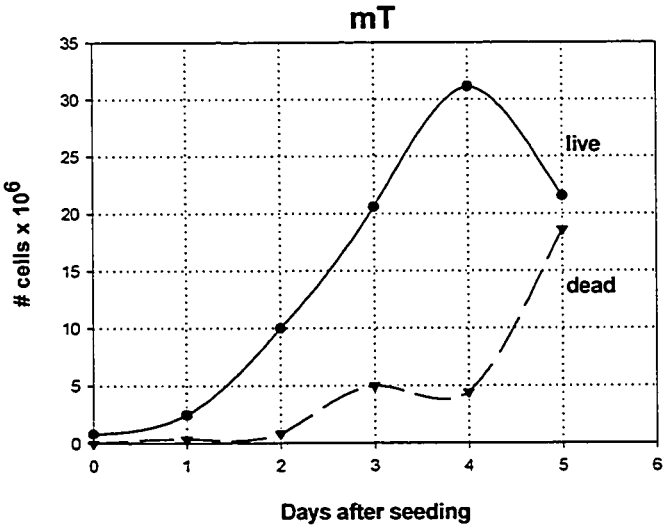
Cell counts (A and B)

Cells were seeded at a density of 80 x 10^4 cells per 80 cm^2 culture flask, and grown in αMEM supplemented with 10% FBS and 250 μg/ml active G418. Every 24 h for 5 days after seeding, cells were collected and counted in the presence of trypan blue. The numbers plotted indicate the total number of live (circles, solid line) and dead (triangles, dotted line) cells, and they represent mean values of 3 separate experiments.

A


Neo
cells x 10^6
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
live
dead
Days after seeding

B


mT
cells x 10^6
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
live
dead
Days after seeding

1.3 Phenotypic Alterations

The morphology of the Neo cells was slightly different than that of the mT cells (Fig. 4A and 4B). The Neo cells were well attached, large and flat. Upon reaching confluence in the culture flask, they remained as a single layer and adopted a polygonal, less round shape (Fig. 4A). On the other hand, the middle T transfected cells were much smaller and elongated. Upon reaching confluence, these cells continued to multiply and grow, forming numerous layers of cells, as well as foci in the flask (Fig. 4B).

One of the features of a transformed phenotype is a cell's ability to maintain anchorage-independent growth (Alberts et al., 1989). In order to assess the ability of these cells to sustain growth in the absence of a solid substratum, approximately 5×10^4 cells were plated in 60 mm culture dishes containing soft agar. 1 ml of fresh growth medium was added to each culture dish once a week. The Neo cells died rapidly within the first few days after plating (Fig. 4C). No viable cells were observed after three weeks. Under the same experimental conditions, the mT cells survived, multiplied and formed colonies, easily seen within one week after plating. After 3 weeks, multiple colonies of mT cells were observed in the soft agar dishes (Fig. 4D).

II. Sensitivity to apoptotic stimuli

Two different stimuli, i.e. the removal of growth factors and treatment with the DNA damaging agent VM26, were used to induce apoptosis in T51B cells. VM26, or teniposide, is a topoisomerase II inhibitor which is commonly used as a chemotherapeutic drug and has

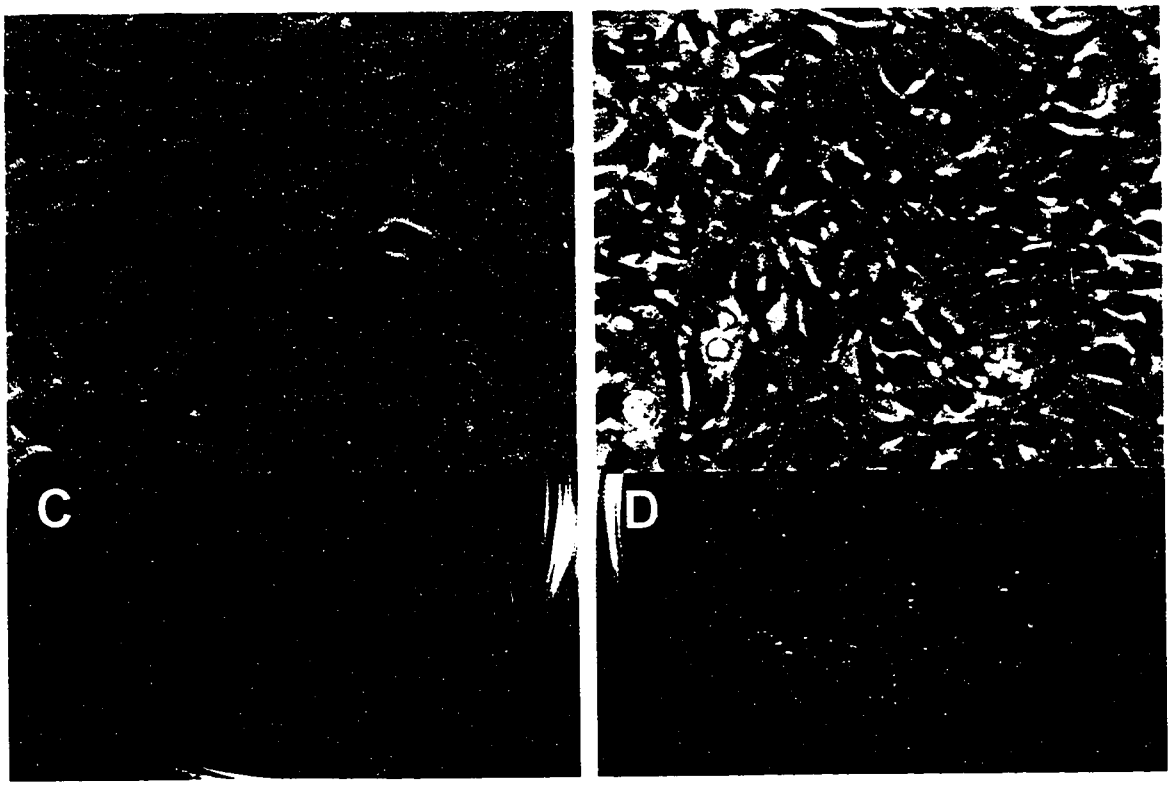
Figure 4: Phenotypic alterations

Phase contrast microscopy (A and B)

Four days after seeding, confluent Neo (A) and mT (B) cells were photographed under phase contrast using the Zeiss IM35 microscope and Polaroid film #55.
Magnification: 150X

Growth in soft agar (C and D)

Cells were seeded in 0.6% agar plates, fed once a week with complete medium, and photographed 3 weeks after seeding using a combination of transmitted and reflected lighting and Polaroid film #55.
Magnification: 2X


C
D
Neo
mT

been shown to induce apoptosis in cultured cells (Tsuruo and Ogawa 1992, Walker et al. 1993, Roy et al. 1992). Apoptosis was assessed both morphologically by Hoechst 33258 staining of cell nuclei, and biochemically by trypan blue exclusion assays, MTT cell viability assays and pulsed field gel analysis for DNA fragmentation.

2.1 Growth factor deprivation

75% confluent cultures were placed in serum-free medium for a period of up to three days and the cells' viability was assessed every day. The morphology of the Neo cells did not change; they remained large, round and attached to the culture flask. Throughout the treatment their behavior remained similar to control, untreated cells. They continued to divide, reached confluence within the first day following treatment and then became quiescent. Between the first and second days of treatment the percentage of trypan blue-positive cells remained between 4% and 16%; no significant increase in cell death was observed until day 3 after treatment (Fig. 5A). At this time 33% of Neo cells stained trypan blue-positive.

The MTT assay, which measures cellular mitochondrial activity (Hansen et al., 1989), was used as another means to assess cell viability. This assay relies on the reduction of a tetrazolium salt to its blue formazan product by NADH and succinate dehydrogenases; therefore it requires intact mitochondria of live cells . The results demonstrated that within one day of serum removal, the mitochondrial activity of Neo cells decreased to approximately 60% of the control level (Fig. 5C). At this time, the cells had become confluent and quiescent. This activity decreased further after the second and third day of

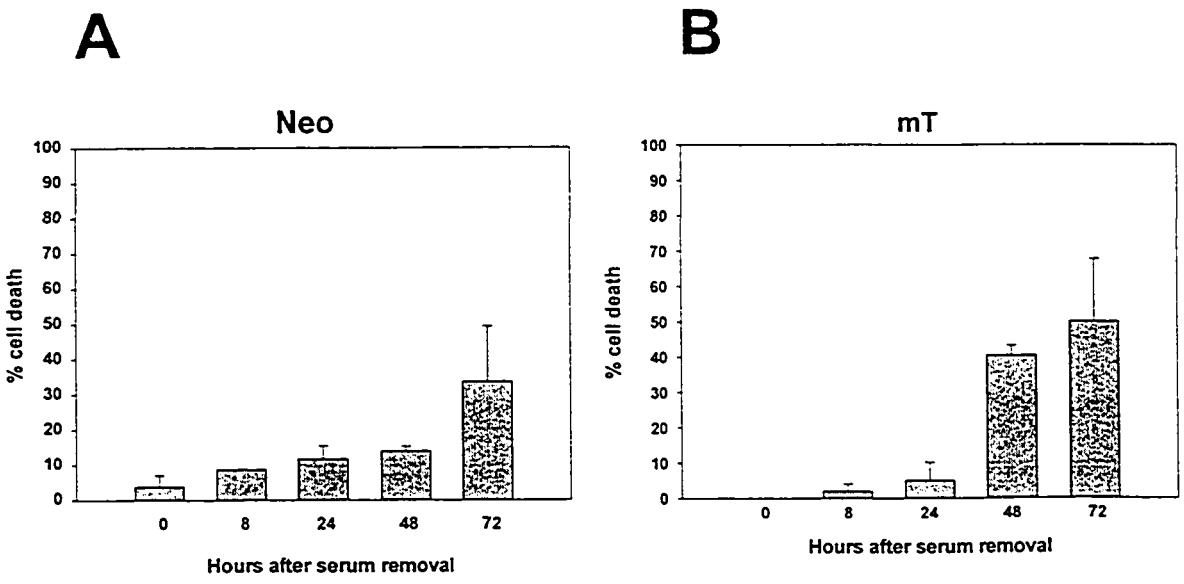
Figure 5: Effects of growth factor deprivation on cell viability

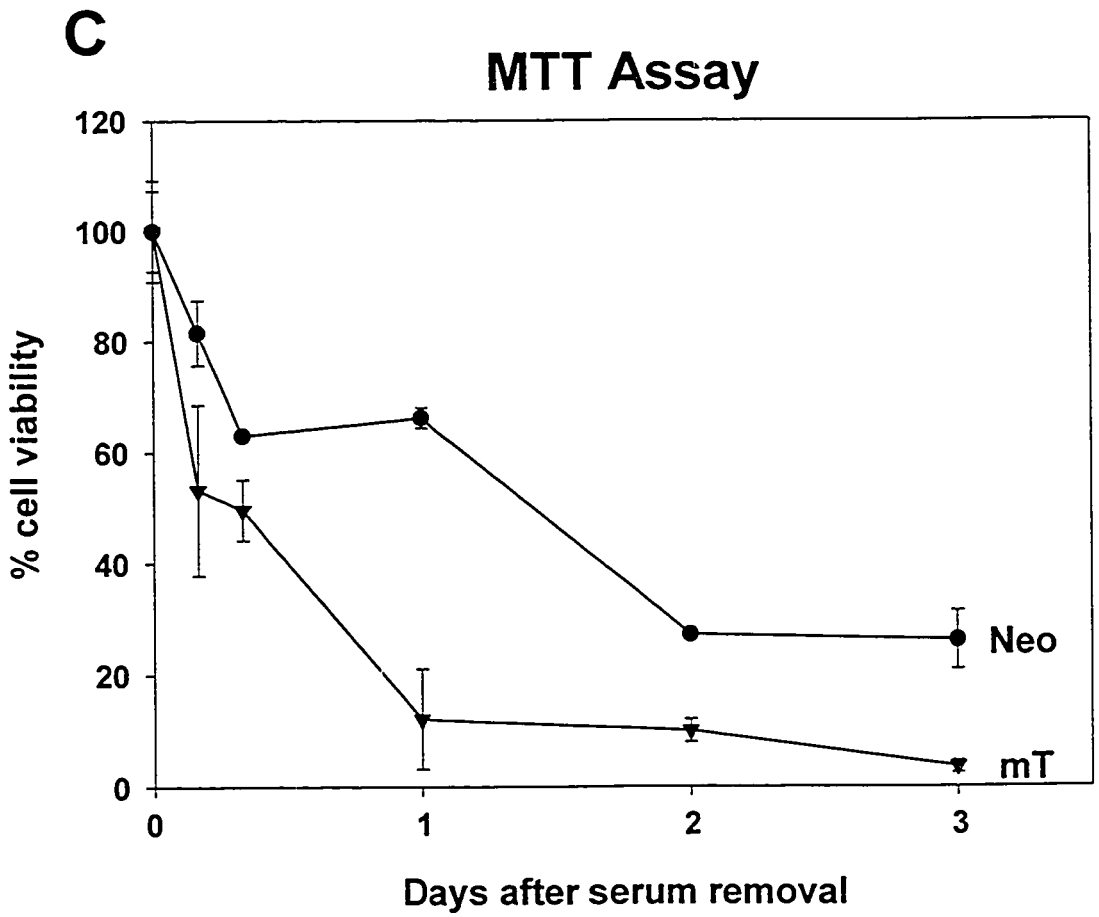
Trypan blue exclusion assay (A and B)

Cells were grown in complete medium to 75% confluence, then placed in serum-free medium for 8 h, 24 h, 48 h and 72 h. Cells were collected and counted in the presence of trypan blue. The numbers plotted indicate mean values ± standard error of the total number of dead cells from 3 separate experiments.
P-value: 0.15

MTT assay (C)

Cells were grown in complete medium in 24-well plates for 24 hours, then placed in serum-free medium for 4 h, 8 h, 24 h, 48 h and 72 h. Viability of Neo (circles) and mT (triangles) cells is expressed as a percentage of control untreated cells. The numbers plotted indicate mean values ± standard error from 3 separate experiments.
P-value: 0.20


A
Neo
% cell death
100
90
80
70
60
50
40
30
20
10
0
0
8
24
48
72
Hours after serum removal
B
mT
% cell death
100
90
80
70
60
50
40
30
20
10
0
0
8
24
48
72
Hours after serum removal


C
MTT Assay
% cell viability
120
100
80
60
40
20
0
0
1
2
3
Days after serum removal
Neo
mT

treatment to approximately 30% of the control value.

In contrast to the Neo cells, the mT cells began to detach from the flask within the first day after the removal of growth factors. The cells stopped proliferating, and they did not reach confluence. In addition, they shrunk as they rounded and detached from the flask. However, no significant increase in the number of trypan blue-positive cells was observed until 48 h following serum deprivation (Fig. 5B). At this time, 40% of mT cells stained blue. Unlike Neo cells, the mT cells showed a sharp drop in mitochondrial activity to approximately 10% of the control level within the first day of serum deprivation (Fig. 5C). The activity remained this low for the duration of the experiment.

Pulsed field gel electrophoresis (PFGE) was performed in order to assess DNA damage. This assay allows the visualization of high molecular weight (HMW) DNA fragments, characteristic of apoptosis, on agarose gels. Serum deprived cells were harvested and embedded in agarose plugs. Slices of the plugs were loaded on an agarose gel, and PFGE was performed (Fig. 6). The results showed that the genomic DNA extracted from Neo cells remained intact for up to 48 h (Fig. 6A). No HMW DNA degradation or DNA laddering was observed during this time and Hoechst 33258-stained Neo cell nuclei had normal morphology (Fig. 6B). The same PFGE analysis of mT cells' DNA demonstrated extensive HMW degradation two days after serum deprivation (Fig. 6C). No significant fragmentation was seen prior to this time point. Staining of the mT nuclei with Hoechst 33258 revealed the appearance of nuclear changes consistent with apoptosis i.e. chromatin collapse and fragmentation into apoptotic bodies (Fig. 6D).

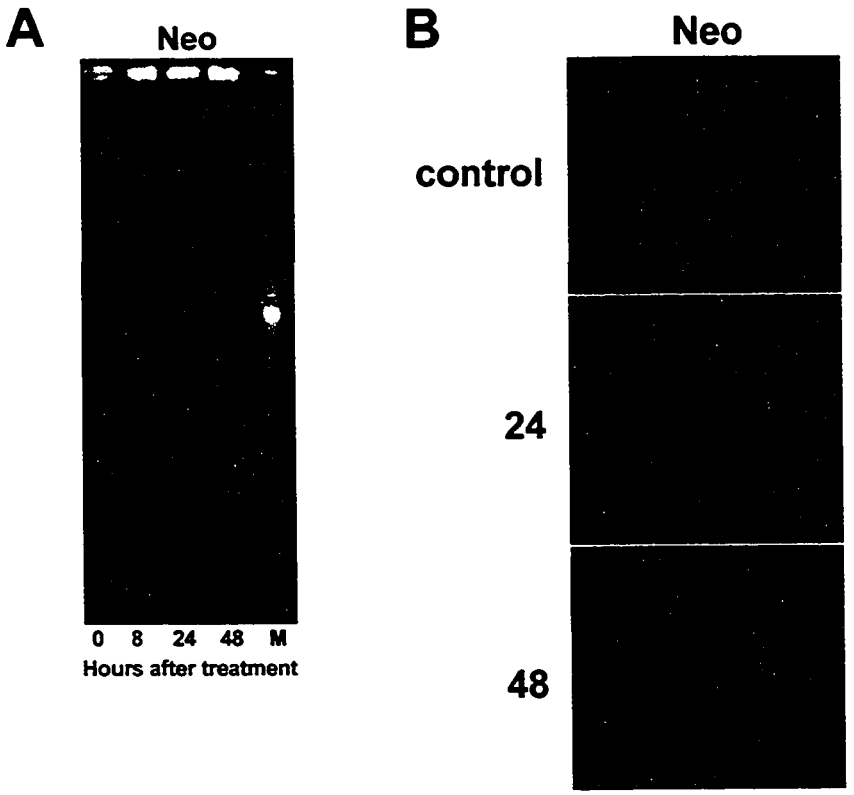
Figure 6: Effects of growth factor deprivation on DNA integrity

Pulsed field gel electrophoresis (A and C)

Cells were grown in complete medium to 75% confluence, then placed in serum-free medium for 8 h, 24 h and 48 h. Cells were harvested and embedded in 1.5% LMP agarose plugs. Slices of the plugs equivalent to 1.25 x 10^6 cells were loaded onto a 0.8% agarose gel and PFGE was carried out. DNA size markers (M), lambda ladder PFG marker and yeast chromosome PFG marker, were separated along with Neo and mT samples, respectively. Gels were stained in ethidium bromide and photographed on a UV transilluminator using Polaroid film #55.

Hoechst 33258 staining (B and D)

Cells were grown on glass cover slips, then placed in serum-free medium for 24 h and 48 h. Nuclei were stained with Hoechst 33258 dye and the cells were observed under an Olympus BX50 microscope equipped with phase and epifluorescence optics and photographed using Kodak Ektachrome film.
Magnification: 500X


A
Neo
0 8 24 48 M
Hours after treatment
B
Neo
control
24
48
C
mT
0 8 24 48 M
Hours after treatment
D
mT
control
24
48

In general, the removal of serum was detrimental to the mT cells but less damaging to the Neo cells. The mT cells could neither survive nor proliferate in the absence of growth factors, and in fact they were dying by apoptosis.

2.2 VM26 treatment

75% confluent cultures were placed in medium containing 10 μM VM26 for a period of up to three days and the cells' viability was assessed every day. Upon addition of the chemotherapeutic drug, the Neo cells stopped proliferating and began to detach from the flask. Many round, floating cells were seen within the first day of treatment. In fact, trypan blue exclusion assays demonstrated that one day after the treatment, 47% of Neo cells were trypan blue-positive, and this number increased up to 63% and 90% by day 2 and 3, respectively (Fig. 7A). The MTT assay showed that the mitochondrial activity of VM26-treated Neo cells dropped steadily, reaching a low level of 10% on the second day after the drug treatment (Fig. 7C).

The VM26-treated mT cells also stopped proliferating, however, for the first two days following the treatment these cells remained attached to the flask and no floating cells were observed. Cell counts in the presence of trypan blue showed that the 24 h VM26 treatment had no effect on the viability of these cells. Cells began to die only on the second and third days of continuous drug exposure, when 30% and 80% of cells stained trypan blue-positive (Fig. 7B). The MTT assay demonstrated that, initially, mitochondrial activity of mT cells dropped to approximately 75% of control within 8 h of drug treatment (Fig. 7C). This, however, was followed by an increase to a level even higher than that of control cells, i.e.

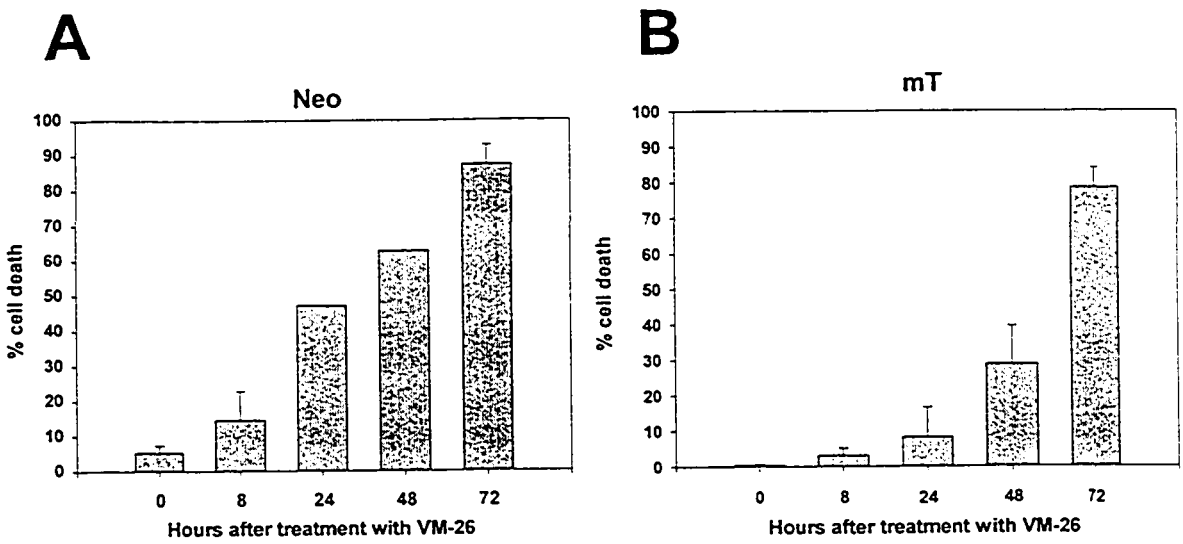
Figure 7: Effects of VM26 treatment on cell viability

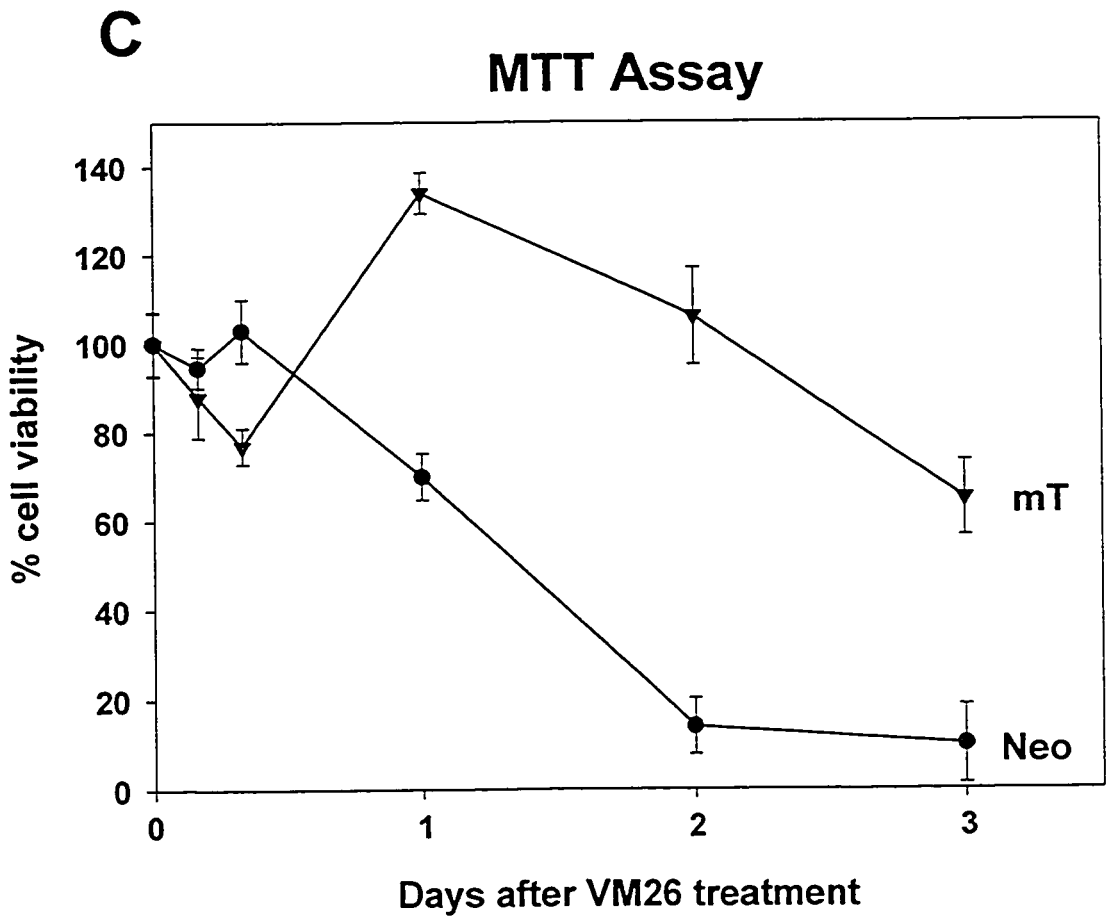
Trypan blue exclusion assay (A and B)

Cells were grown in complete medium to 75% confluence, then placed in medium containing 10 μM VM26 for 8 h, 24 h, 48 h and 72 h. Cells were collected and counted in the presence of trypan blue. The numbers plotted indicate mean values ± standard error of the total number of dead cells from 3 separate experiments.
P-value: 0.03

MTT assay (C)

Cells were grown in complete medium in 24-well plates for 24 hours, then placed in medium containing 10 μM VM26 for 4 h, 8 h, 24 h, 48 h and 72 h. Viability of Neo (circles) and mT (triangles) cells is expressed as a percentage of control untreated cells. The numbers plotted indicate mean values ± standard error from 3 separate experiments.
P-value: 0.10


A
Neo
% cell death
100
90
80
70
60
50
40
30
20
10
0
0
8
24
48
72
Hours after treatment with VM-26
B
mT
% cell death
100
90
80
70
60
50
40
30
20
10
0
0
8
24
48
72
Hours after treatment with VM-26


C
MTT Assay
% cell viability
140
120
100
80
60
40
20
0
0
1
2
3
Days after VM26 treatment
mT
Neo

140% of control value. This activity never dropped below approximately 70% of control, even on the third day of treatment.

PFGE results demonstrated that VM26-treated Neo cells had extensive DNA damage. This was evident within 8 h of drug exposure. In addition to 300 kb DNA fragments, 50 kb and even smaller fragments were observed in each sample analyzed (Fig. 8A). The nuclei of Neo cells had a typical apoptotic morphology, i.e. chromatin condensation and margination against the nuclear periphery (Fig. 8B). The PFGE analysis of genomic DNA from mT cells revealed much less extensive HMW damage caused by VM26. In fact, 300 kb but not 50 kb fragmentation was seen at 8 h (Fig. 8C). Hoechst 33258 staining of mT cells showed that the majority of cells displayed normal nuclear morphology, even at day 2 after the treatment (Fig. 8D). Although some 50 kb and smaller fragments were detected, neither Neo nor mT cells produced a DNA ladder.

III. Apoptosis-associated changes in gene expression

Of the many genes implicated in the cellular response to DNA damage, the tumor suppressor *p53* occupies a central role (Götz and Montenarh 1995, Levine 1997). This protein participates in genome surveillance for DNA damage and when damage is detected, p53 has been shown to initiate either GADD45-mediated growth arrest or Bax-induced apoptosis (Levine 1997). In order to determine if the apoptotic stimuli triggered a gene - directed (i.e. *p53*, *GADD45* and *Bax*) response, Northern blotting was performed following either growth factor deprivation or treatment with VM26.

Figure 8: Effects of VM26 treatment on DNA integrity

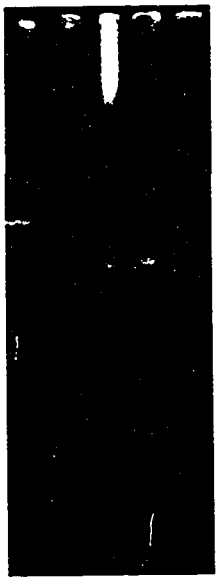
Pulsed field gel electrophoresis (A and C)

Cells were grown in complete medium to 75% confluence, then placed in medium containing 10 μM VM26 for 8 h, 24 h and 48 h. Cells were harvested and embedded in 1.5% LMP agarose plugs. Slices of the plugs equivalent to 1.25 x 10^6 cells were loaded onto a 0.8% agarose gel and PFGE was carried out. DNA size markers (M), yeast chromosome PFG marker and lambda ladder PFG marker, were separated along with Neo and mT samples, respectively. Gels were stained in ethidium bromide and photographed on a UV transilluminator using Polaroid film #55.

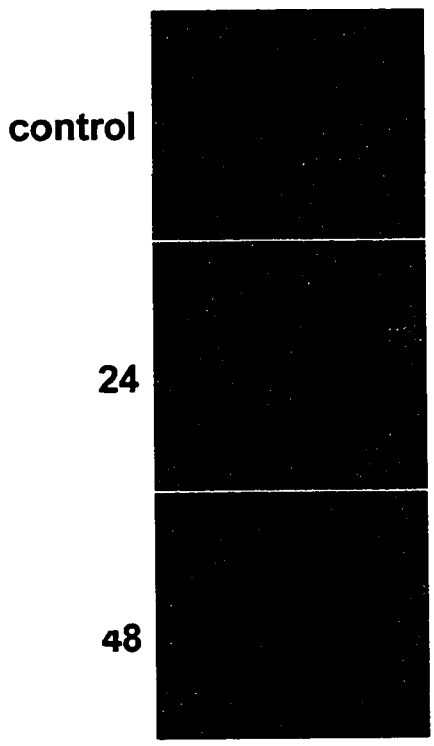
Hoechst 33258 staining (B and D)

Cells were grown on glass cover slips, then placed in medium containing 10 μM VM26 for 24 h and 48 h. Nuclei were stained with Hoechst 33258 dye and the cells were observed under an Olympus BX50 microscope equipped with phase and epifluorescence optics and photographed using Kodak Ektachrome film.
Magnification: 500X

A


Neo
M 0 8 24 48
Hours after treatment

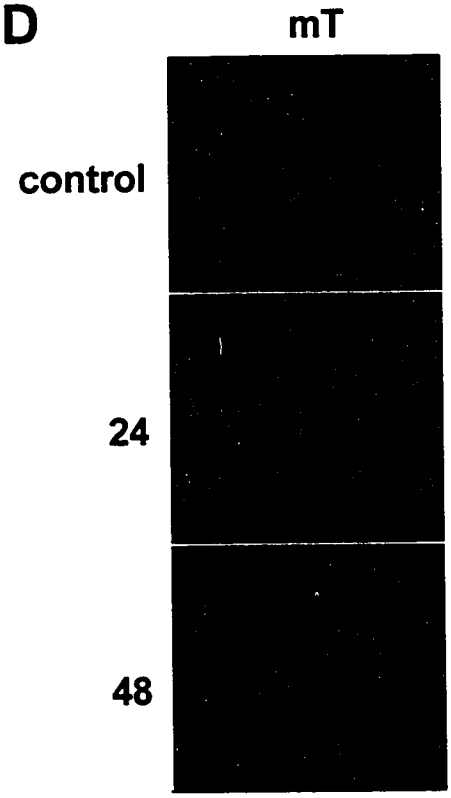
B


Neo
control
24
48

C


mT
M 0 8 24 48
Hours after treatment

D


mT
control
24
48

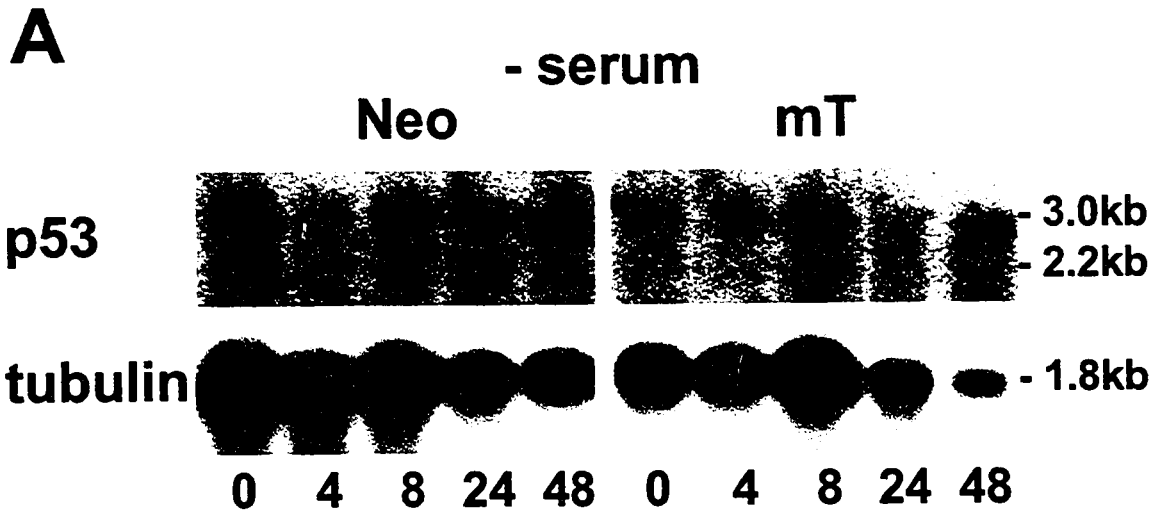
Typically, differences in the amount of mRNA loaded onto the gel are compared to and corrected according to the expression of a housekeeping gene, such as *α-tubulin.* With this intent, the Northern blots were probed with *α-tubulin.* However, it became apparent that this gene was downregulated during apoptosis, particulary following VM26 treatment. Since this type of data quantitation was not possible, care was taken to always load identical amounts of mRNA, as verified by absorbance at 260nm.

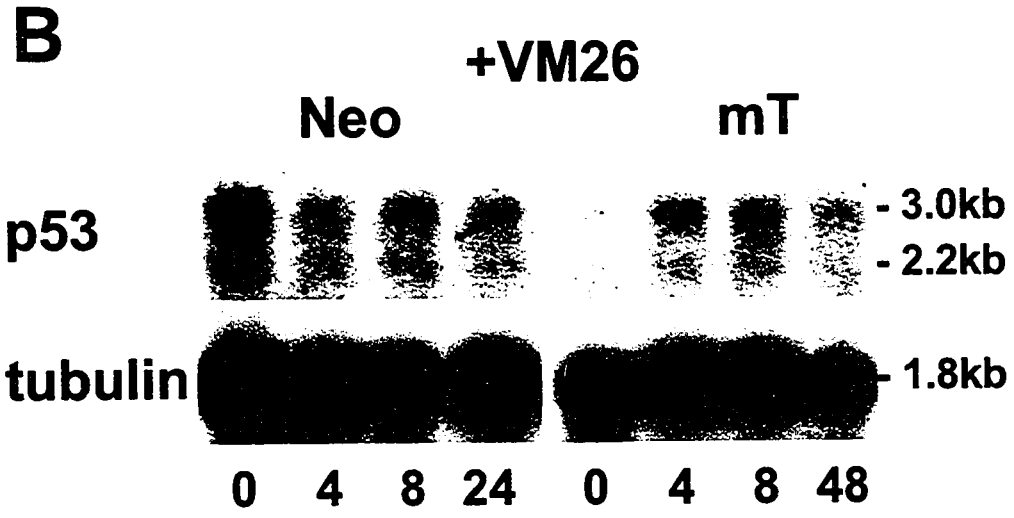
3.1 *p53*

Two mRNA species, 3.0 kb and 2.2 kb, were detected in Neo cells and only one (i.e. 3.0 kb) in mT cells (Fig. 9). The expression of *p53* was lower in mT cells than in Neo cells. The message was significantly downregulated in Neo cells and remained unchanged in mT cells after they were placed in serum-free medium (Fig. 9A). For the duration of the 48 h experiment, the *p53* signal remained very low in both cell lines. Following the addition of 10 μM VM26 to Neo cells, the expression of *p53* decreased immediately, as it did in serum-free conditions (Fig. 9A) and remained at this lower level up to 24 h (Fig. 9B). At no time during either treatment did the level of *p53* increase above that of control cells. Immediately following the addition of VM26, the *p53* signal in mT cells increased to a level higher than that in untreated cells and remained higher for the entire 48 h drug treatment (Fig. 9B).

Fig. 9: Changes in *p53* expression

mRNA was isolated and analyzed by Northern blotting after 4 h, 8 h, 24 h and 48 h of either serum deprivation (A) or treatment with 10 μM VM26 (B). Approximately 400ηg mRNA was separated on a 1% agarose denaturing gel and transferred onto a nylon membrane. The blot was hybridized with ^{32}P-labelled 2.0 kb BamH1 restriction fragments of *p53* and 1.6 kb Pst1 restriction fragments of *α-tubulin*, and exposed to Kodak X-OMAT film. The film was developed, then scanned using a HPScanJet 2CX.


A
- serum
Neo
mT
p53
tubulin
- 3.0kb
- 2.2kb
- 1.8kb
0 4 8 24 48 0 4 8 24 48


B
+VM26
Neo
mT
p53
tubulin
- 3.0kb
- 2.2kb
- 1.8kb
0 4 8 24 0 4 8 48

3.2 *GADD45*

The 1.4 kb *GADD45* transcript was expressed both in control Neo, and control mT cells (Fig. 10). In Neo cells the *GADD45* message did not change significantly during the 48 h of serum deprivation (Fig. 10A). However, the message was induced in mT cells, but only 2 days after the removal of growth factors (Fig. 10A). A significant induction of *GADD45* mRNA was seen in both cell lines in response to VM26 treatment (Fig. 10B). The elevated expression of this message was evident even after 24 h and 48 h of the drug treatment.

3.3 *Bax*

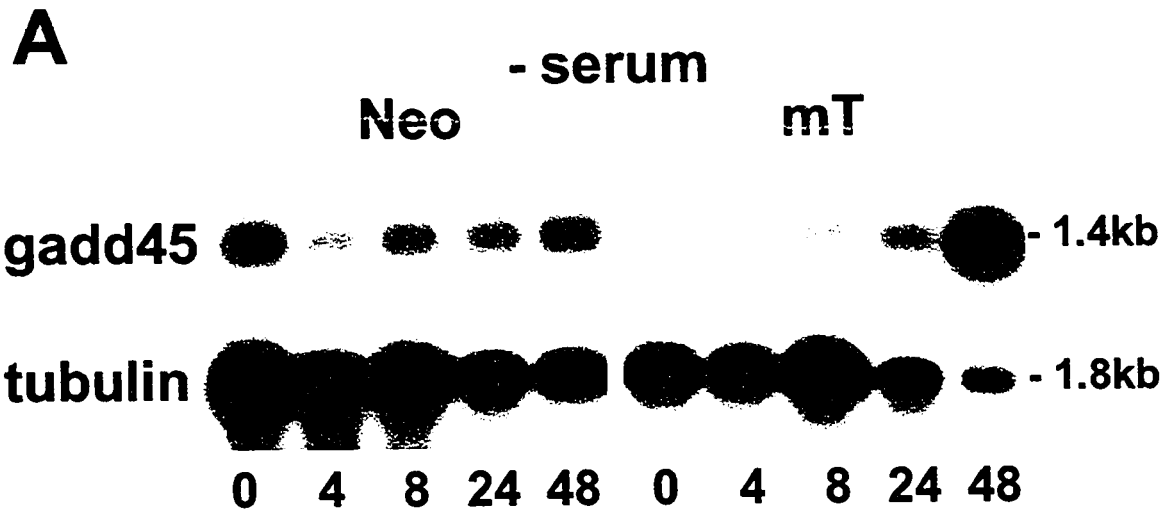
Restriction fragments of *Bax* cDNA hybridized to 1.5 kb and 1.0 kb mRNA species in both cell lines (Fig. 11). The level of the 1.5 kb message was higher than the 1.0 kb. Described below, alterations in *Bax* expression reflected the level of the 1.5 kb mRNA. Serum deprivation resulted in the downregulation of *Bax* in Neo cells and upregulation of the signal in mT cells, evident by 48 h (Fig. 11A). The expression of *Bax* did not change in Neo cells exposed to VM26 for 24 h (Fig. 11B). However, the *Bax* message was induced in the drug-treated mT cells and remained high for up to 48 h of the treatment (Fig. 11B).

IV. Apoptosis-associated changes in cellular proteins

To assess specific changes in cellular protein levels correlated with responses to apoptotic stimuli, both cell lines were analyzed by immunofluorescence staining for a variety

Fig. 10: Changes in *GADD45* expression

mRNA was isolated and analyzed by Northern blotting after 4 h, 8 h, 24 h and 48 h of either serum deprivation (A) or treatment with 10 μM VM26 (B). Approximately 400ηg mRNA was separated on a 1% agarose denaturing gel and transferred onto a nylon membrane. The blot was hybridized with ^{32}P-labelled 1.2 kb Sac1/Kpn1 restriction fragments of *GADD45*, and 1.6 kb Pst1 restriction fragments of *α-tubulin*, and exposed to Kodak X-OMAT film. The film was developed, then scanned using a HPScanJet 2CX.


A
- serum
Neo
mT
gadd45
- 1.4kb
tubulin
- 1.8kb
0 4 8 24 48 0 4 8 24 48

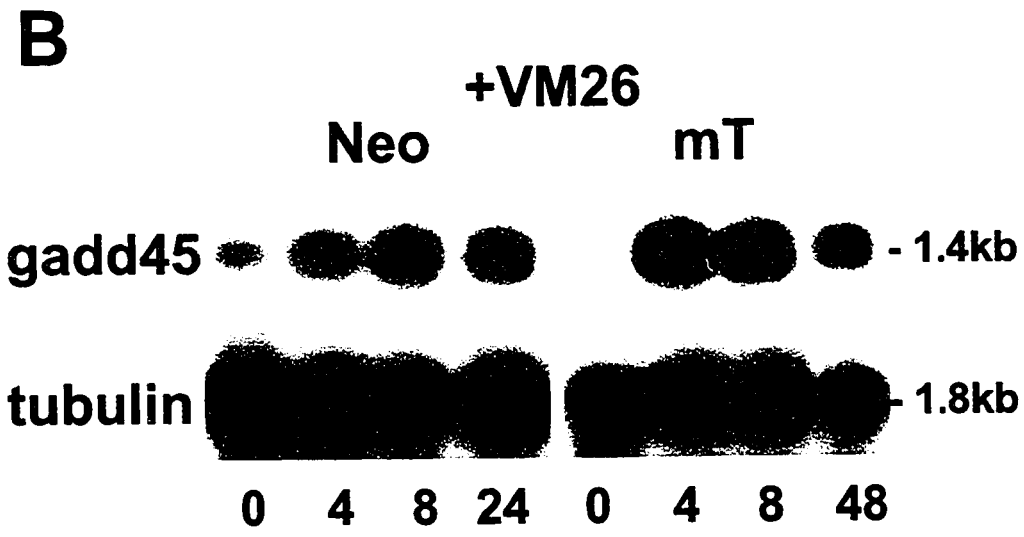
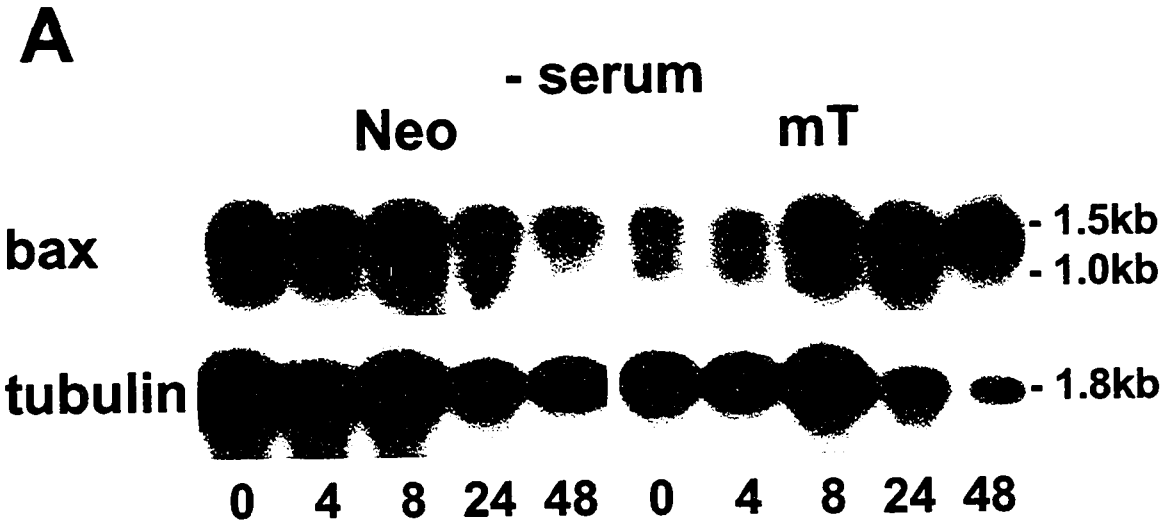
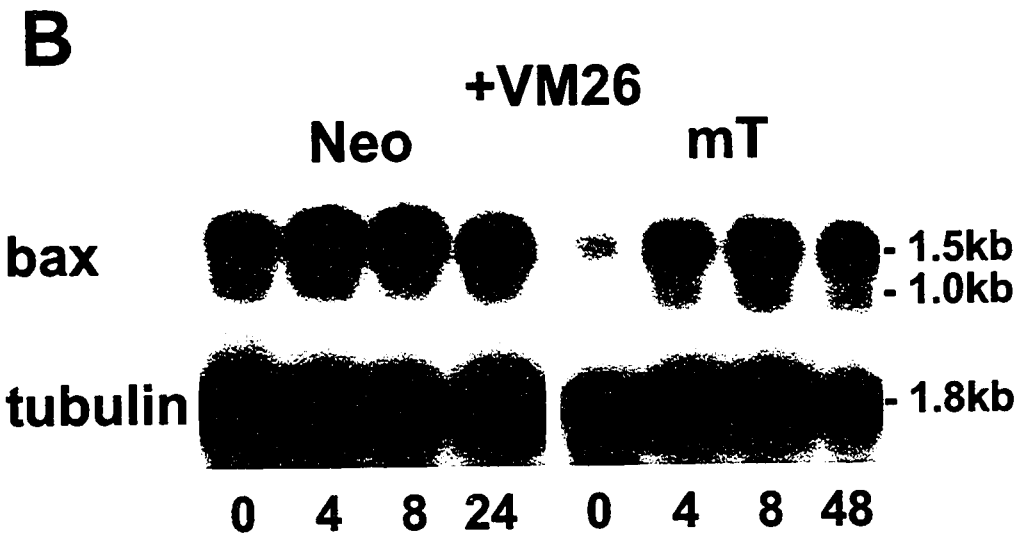

B
+VM26
Neo
mT
gadd45
- 1.4kb
tubulin
- 1.8kb
0 4 8 24 0 4 8 48

Fig. 11: Changes in *Bax* expression

mRNA was isolated and analyzed by Northern blotting after 4 h, 8 h, 24 h and 48 h of either serum deprivation (A) or treatment with 10 μM VM26 (B). Approximately 400ηg mRNA was separated on a 1% agarose denaturing gel and transferred onto a nylon membrane. The blot was hybridized with ^{32}P-labelled 0.94 kb EcoR1 restriction fragments of *Bax* and 1.6 kb Pst1 restriction fragments of *α-tubulin*, and exposed to Kodak X-OMAT film. The film was developed, then scanned using a HPScanJet 2CX.


A
- serum
Neo
mT
bax
tubulin
- 1.5kb
- 1.0kb
- 1.8kb
0 4 8 24 48 0 4 8 24 48


B
+VM26
Neo
mT
bax
tubulin
- 1.5kb
- 1.0kb
- 1.8kb
0 4 8 24 0 4 8 48

of antigens, including aMAPK, GADD45, the p53-dependent cell cycle regulator $p21^{waf-1}$ and the DNA-dependent protein kinase (DNA-PK).

4.1 aMAPK

As shown in Fig. 2, mT expression led to the constitutive activation of MAPK. To determine whether this signaling pathway participated in mediation of apoptotic responses, cells were stained with anti-aMAPK and analyzed by microscopy.

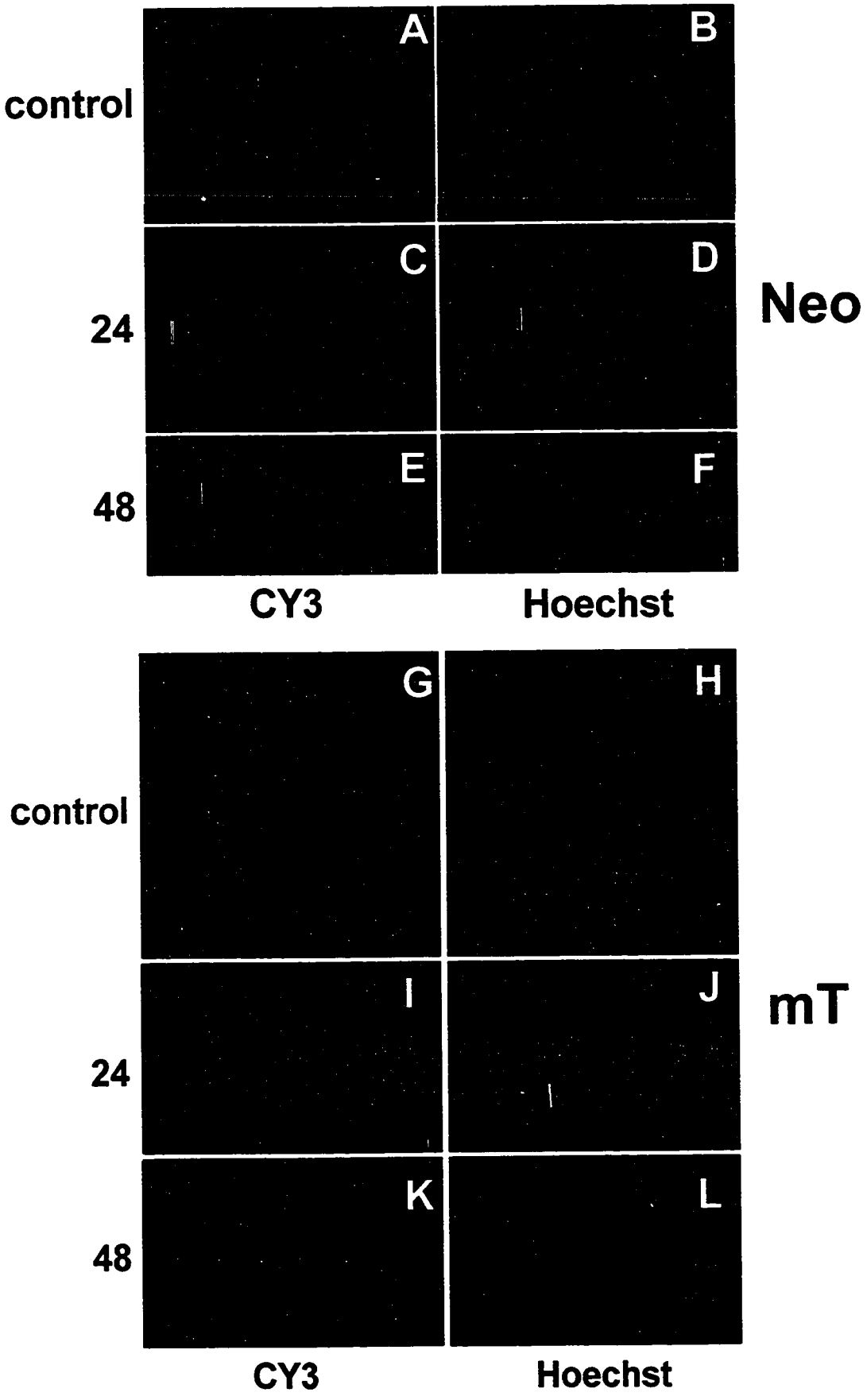
In control Neo cells, the aMAPK was detected exclusively in mitotic cells where the protein was closely associated with chromatin (Fig. 12A-F). In the remaining cell population, the aMAPK signal was undetectable. As shown in Fig. 6, the Neo cells were able to survive in the absence of growth factors for up to 3 days and the pattern of aMAPK staining did not change during the treatment (Fig. 12); the protein was still detected only in dividing cells (Fig. 12C-E).

In contrast, control mT cells showed the presence of aMAPK in the entire cell population. A strong fluorescence signal was detected in both the cytosol and the nuclei of these cells (Fig. 12G-L). The mT cells, which were very sensitive to serum deprivation, demonstrated a significant decrease in the aMAPK signal in response to the treatment. There was no longer cytoplasmic staining for aMAPK 24 h after serum withdrawal (Fig. 12I-J) and the nuclear signal was much weaker. The aMAPK signal was not detectable in cells showing fully developed apoptotic morphology (Fig. 12K-L).

The VM26 treatment was detrimental to the Neo cells and a high percentage of dead

Figure 12: Changes in aMAPK following removal of growth factors

Neo (A to F) and mT (G to L) cells were grown on glass cover slips and placed in serum-free medium for 24 h and 48 h. Cells were immunostained with anti-phosphorylated MAPK rabbit polyclonal antibody and CY3-conjugated donkey anti-rabbit secondary antibody (A, C, E, G, I, K). The nuclei were counterstained with Hoechst 33258 dye (B, D, F, H, J, L) and the cells were observed under Olympus BX50 microscope equipped with phase and epifluorescence optics and photographed using Kodak Ektachrome film.
Magnification: 810X


A
B
control
C
D
24
Neo
E
F
48
CY3
Hoechst
G
H
control
I
J
24
mT
K
L
48
CY3
Hoechst

cells was seen 24 h and 48 h after drug exposure. Although no mitotic cells were seen after VM26 treatment, many aMAPK-positive cells were detected in this population (Fig. 13A-F). However, Hoechst 33258 staining of nuclei revealed that all of these cells had apoptotic morphology (Fig. 13D-F). The aMAPK signal in VM26-resistant mT cells was noticeably stronger in cells exposed to the drug than in untreated cells (Fig. 13G-L). In particular the nuclear staining was more prominent, as shown in cells treated with VM26 for 24 h and 48 h (Fig. 13I and K). The nuclear morphology of these cells still appeared to be normal (Fig. 13J and L).

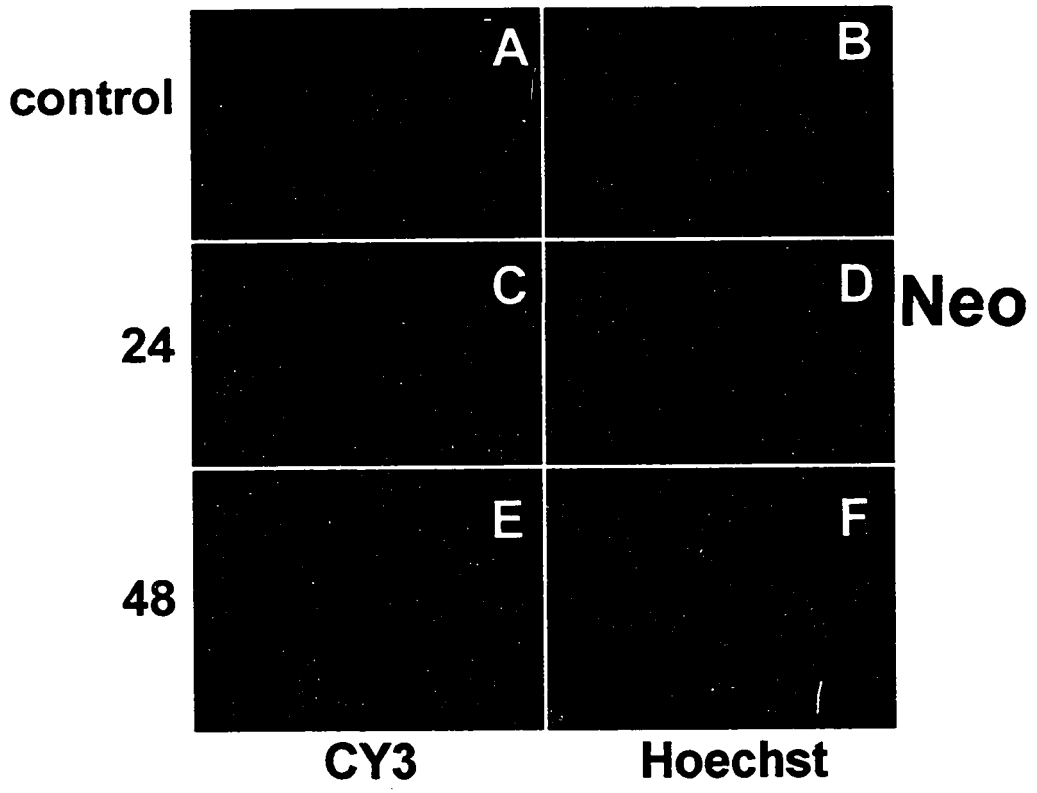
4.2 GADD45

As shown in Fig. 9, the *GADD45* message was detected by Northern blotting in both cell lines. For protein detection, we used rabbit polyclonal antibodies raised against the full amino acid sequence of human GADD45 protein (Fornace 1992). GADD45 was detected in control Neo as well as in control mT cells (Fig. 14). In both cell lines, the immunofluorescence signal was detected exclusively in the nuclei. 24 h after serum deprivation, the amount of GADD45 protein decreased to a barely detectable level in both Neo and mT cells (Fig. 14C and 14I). 48 h after serum withdrawal, very few Neo cells stained with the anti-GADD45 antibody (Fig. 14E). However, the few mT cells which were able to survive 48 h serum deprivation had very high levels of the protein (Fig. 14K).

Following treatment with VM26, the level of GADD45 in both cell lines decreased in the first 24 h (Fig. 15C and 15I). However, after 48 h of drug exposure the GADD45

Figure 13: Changes in aMAPK following VM26 treatment

Neo (A to F) and mT (G to L) cells were grown on glass cover slips and placed in medium containing 10 μM VM26 (G to L) for 24 h and 48 h. Cells were immunostained with anti-phosphorylated MAPK rabbit polyclonal antibody and CY3-conjugated donkey anti-rabbit secondary antibody (A, C, E, G, I, K). The nuclei were counterstained with Hoechst 33258 dye (B, D, F, H, J, L) and the cells were observed under Olympus BX50 microscope equipped with phase and epifluorescence optics and photographed using Kodak Ektachrome film.
Magnification: 810X


control
A
B
C
D
Neo
24
E
F
48
CY3
Hoechst

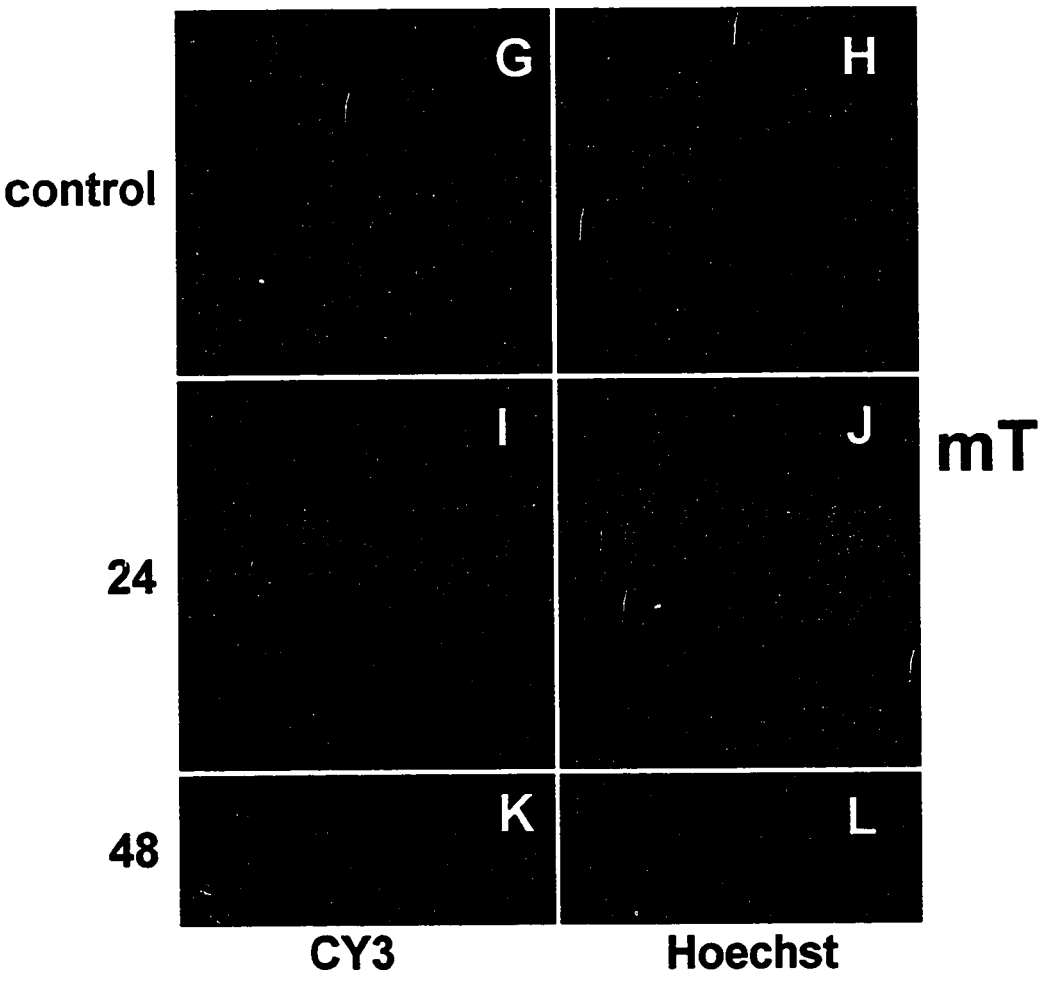
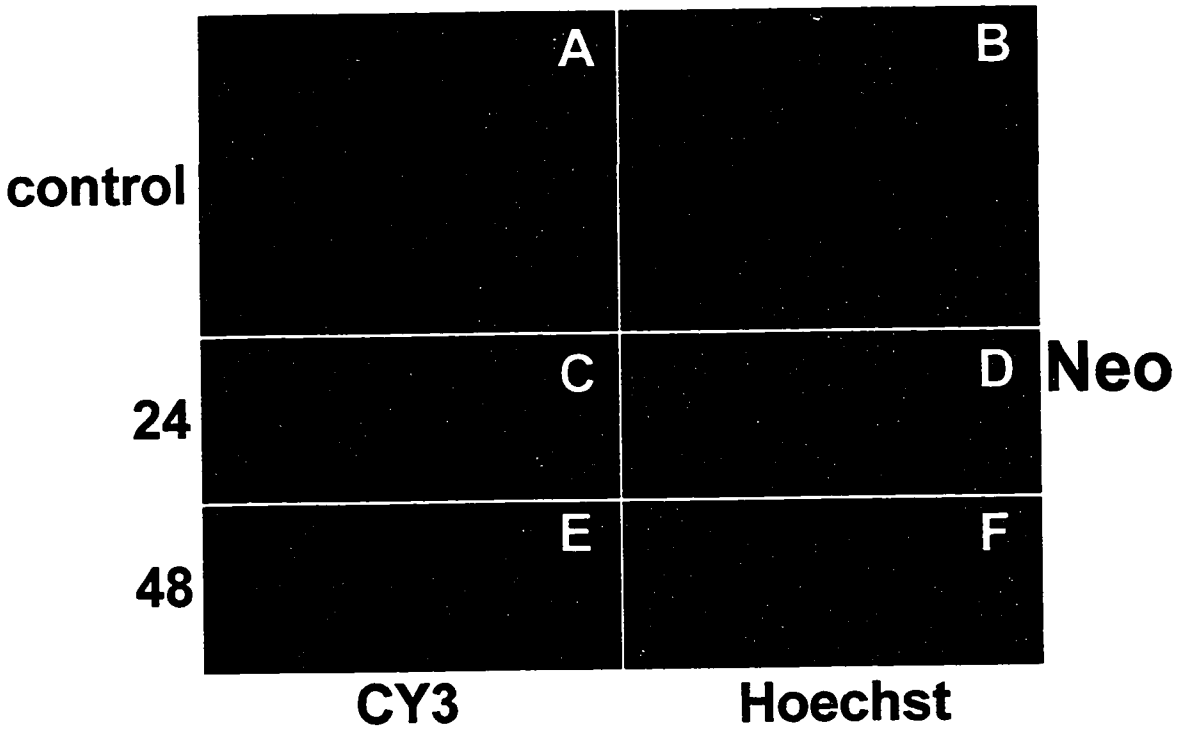

control
G
H
I
J
mT
24
K
L
48
CY3
Hoechst

Figure 14: Changes in GADD45 following removal of growth factors

Neo (A to F) and mT (G to L) cells were grown on glass cover slips and placed in serum-free medium for 24 h and 48 h. Cells were immunostained with anti-GADD45 rabbit polyclonal antibody and CY3-conjugated donkey anti-rabbit secondary antibody (A, C, E, G, I, K). The nuclei were counterstained with Hoechst 33258 dye (B, D, F, H, J, L) and the cells were observed under Olympus BX50 microscope equipped with phase and epifluorescence optics and photographed using Kodak Ektachrome film.
Magnification: 650X


A
B
control
C
D
Neo
24
E
F
48
CY3
Hoechst

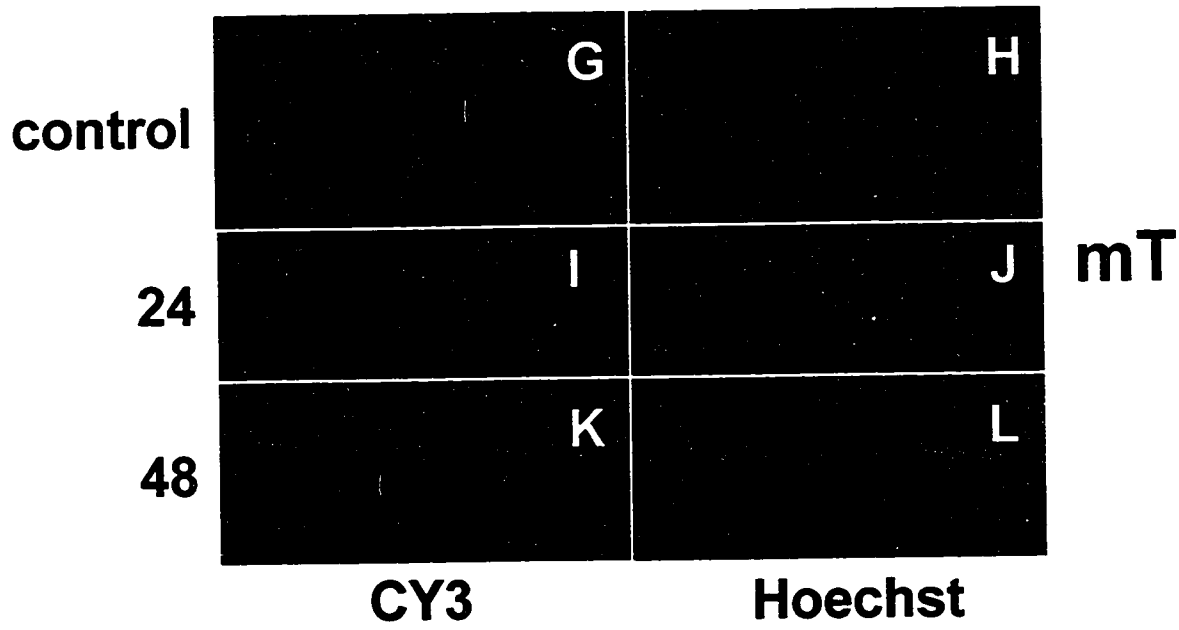
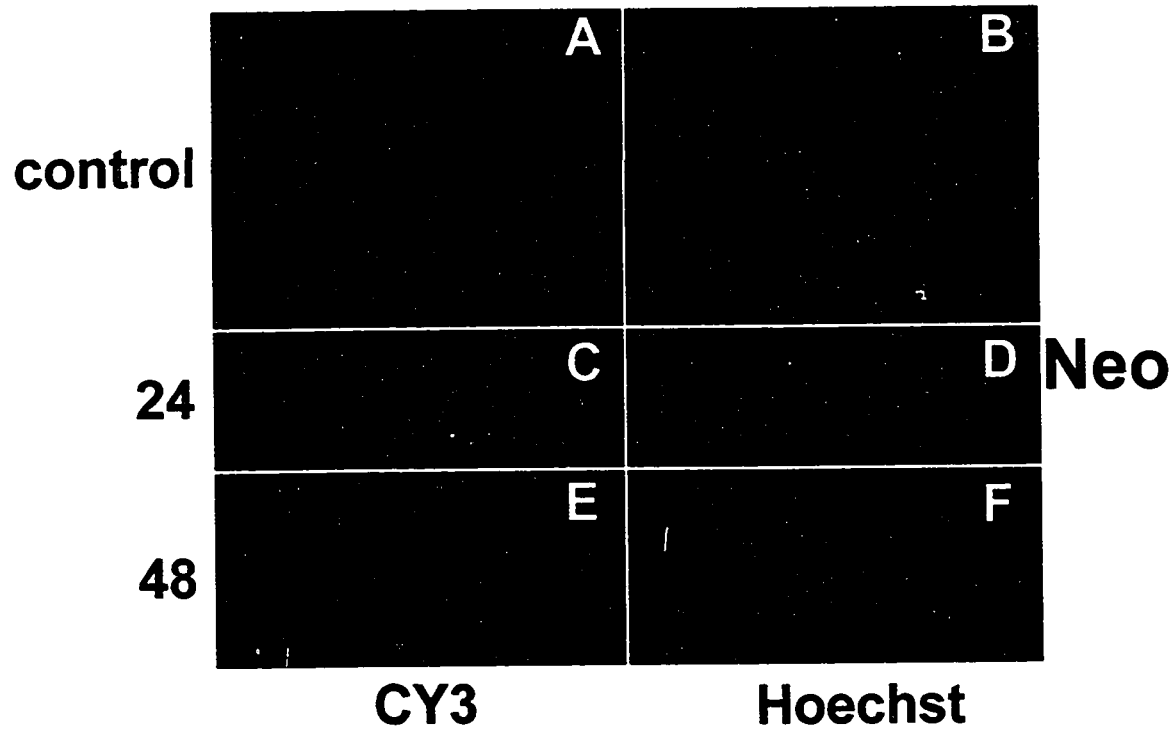

G
H
control
I
J
mT
24
K
L
48
CY3
Hoechst

Figure 15: Changes in GADD45 following VM26 treatment

Neo (A to F) and mT (G to L) cells were grown on glass cover slips and placed in medium containing 10 μM VM26 (G to L) for 24 h and 48 h. Cells were immunostained with anti-GADD45 rabbit polyclonal antibody and CY3-conjugated donkey anti-rabbit secondary antibody (A, C, E, G, I, K). The nuclei were counterstained with Hoechst 33258 dye (B, D, F, H, J, L) and the cells were observed under Olympus BX50 microscope equipped with phase and epifluorescence optics and photographed using Kodak Ektachrome film. Magnification: 650X


A
B
control
C
D
Neo
24
E
F
48
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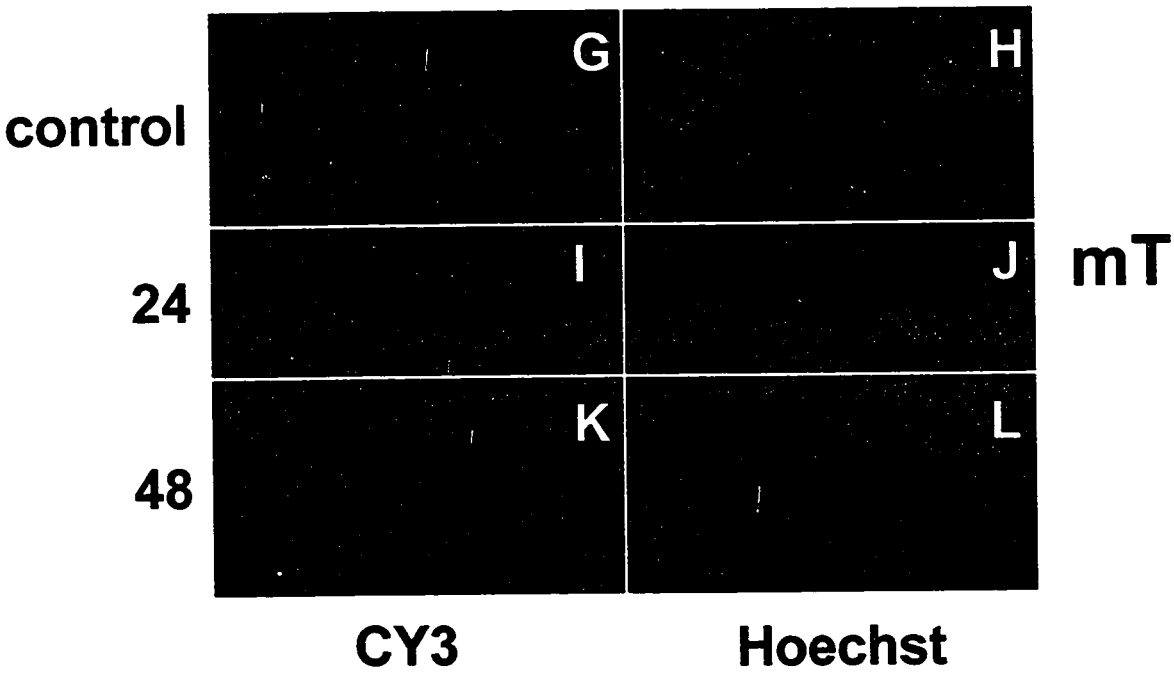
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signal in both surviving Neo and mT cells increased to a level higher than control (Fig. 15E and 15K).

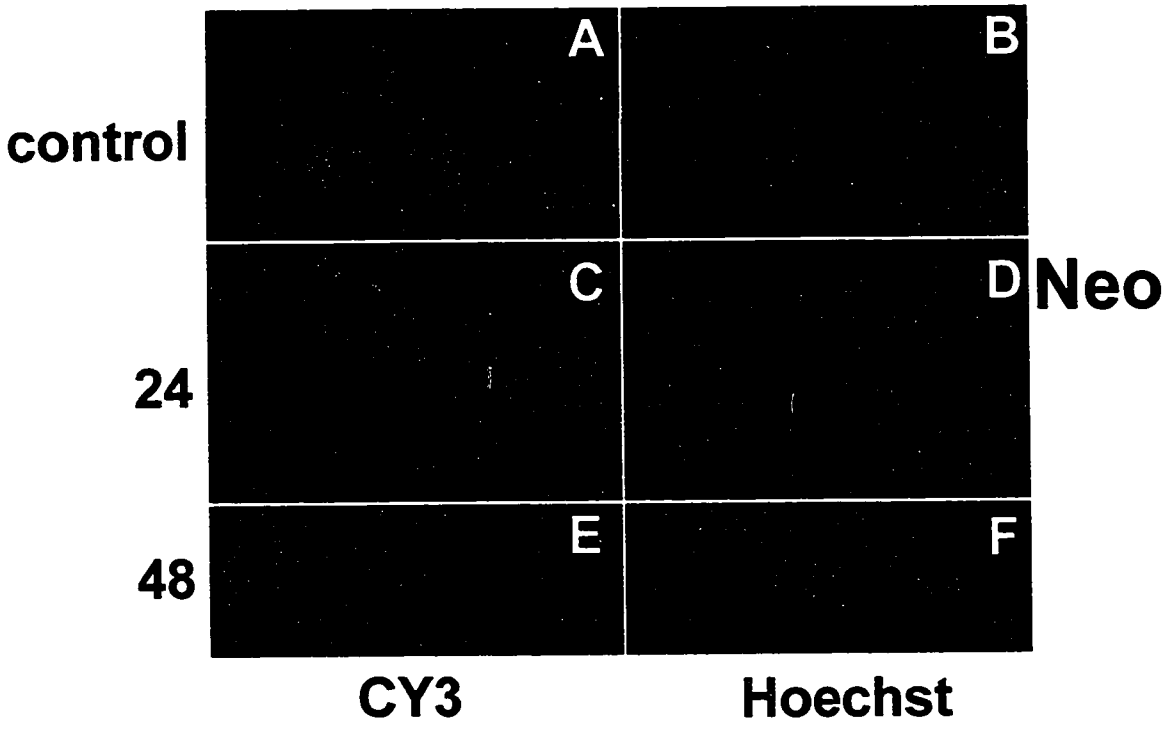
4.3 $p21^{waf-1}$

The p21 protein is another p53-dependent transcript involved in cell cycle arrest, DNA replication and repair functions. However, its role in cellular responses to apoptotic stimuli has not yet been examined. In this study, cells were induced to undergo apoptosis and changes in p21 were analyzed by microscopy after cells were stained with antibodies raised against amino acids 146-164 of the carboxy terminus of human p21 (Harper et al. 1993).

Immunofluorescence staining of p21 in Neo control cells revealed its localization in a few discrete areas in the nuclei (Fig. 16A). These well defined, compact domains stained very brightly. The staining did not appear in nucleoli and there was no evidence of p21 in the cytosol. The control mT cells also showed punctate distribution of p21 within their nuclei, but a low level of diffuse staining throughout the nucleoplasm was also present (Fig. 16G). Within 24 h of serum deprivation, a redistribution of p21 was observed in some Neo cells (Fig. 16C). The cells that showed reorganized p21 had a diffuse, bright signal within their nuclei. No cytosolic staining was seen. The remaining cells showed the same pattern of p21 as in control cells (i.e. punctate staining). Both patterns were seen up to 48 h of serum deprivation but the proportion of cells with diffuse staining steadily increased (Fig. 16E). In the mT cells, which were sensitive to growth factor deprivation, p21 protein level was

Figure 16: Changes in p21 following removal of growth factors

Neo (A to F) and mT (G to L) cells were grown on glass cover slips and placed in serum-free medium for 24 h and 48 h. Cells were immunostained with anti-p21 rabbit polyclonal antibody and CY3-conjugated donkey anti-rabbit secondary antibody (A, C, E, G, I, K). The nuclei were counterstained with Hoechst 33258 dye (B, D, F, H, J, L) and the cells were observed under Olympus BX50 microscope equipped with phase and epifluorescence optics and photographed using Kodak Ektachrome film.
Magnification: 650X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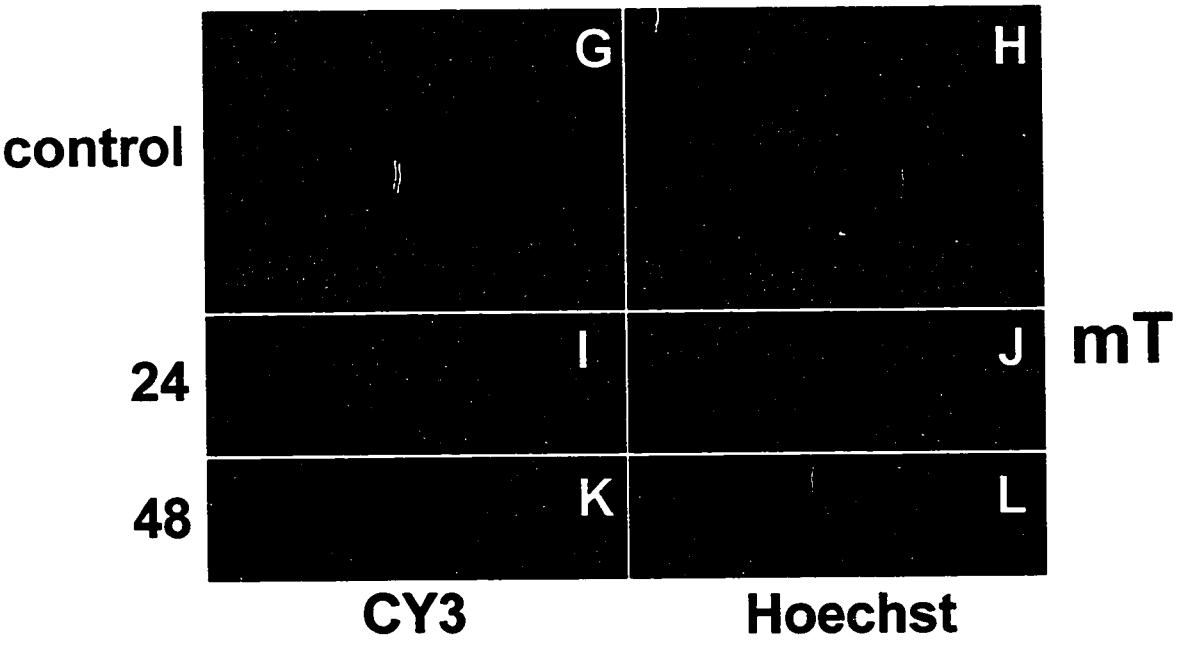
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lower than in control at 24 h and 48 h after serum removal (Fig. 16I and 16K) . Diffuse nuclear staining was seen in these surviving cells, but it was not as bright as in Neo cells.

24 h following the addition of VM26, the p21 signal in the drug-sensitive Neo cells decreased significantly to a barely detectable level (Fig. 17C). The protein was constrained to the nucleus, but the precise sub-nuclear organization of p21 was difficult to determine. However, after 48 h in VM26, p21 staining in the few surviving cells showed an intense, diffuse nuclear signal (Fig. 17E). After the addition of VM26 to mT cells, changes in the p21 signal were detected by 24 h (Fig. 17G). Expression increased to a higher level than control cells and redistribution of the protein was evident in the entire cell population. However, by 48 h of drug exposure, the signal had become almost undetectable (Fig. 17K).

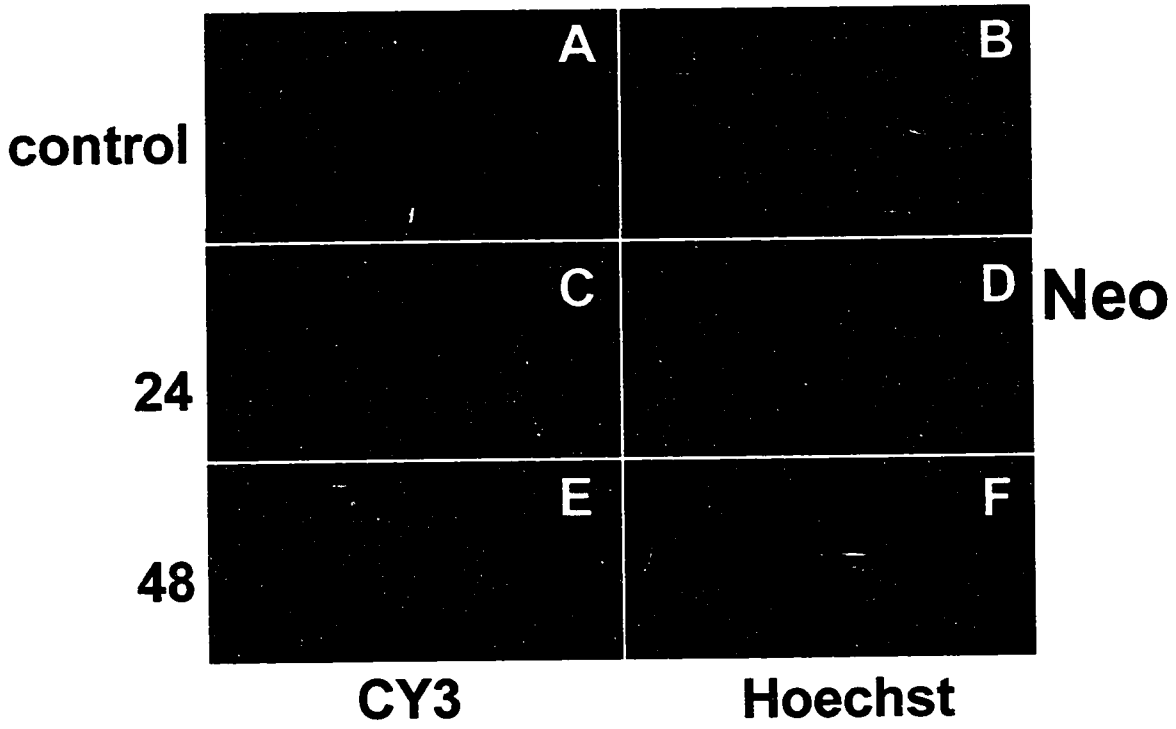
4.4 DNA-dependent Protein Kinase

DNA-dependent protein kinase (DNA-PK) is a nuclear serine/threonine kinase that is activated by DNA double-strand breaks (DSB). It is a component of the DNA DSB repair apparatus. This protein was examined in both cell lines following induction of apoptosis. Antibodies used for immunodetection were raised against a bacterially expressed 16 kDa polypeptide of DNA-PK (Lees-Miller 1996).

Immunofluorescence staining of DNA-PK in control Neo cells demonstrated diffuse staining of the protein in the nucleus (Fig. 18A). In addition, staining revealed one filamentous, perinuclear structure per cell (indicated by white arrow heads on Figs. 18 and 19). This structure did not have a clearly discernible shape and its subcellular organization

Figure 17: Changes in p21 following VM26 treatment

Neo (A to F) and mT (G to L) cells were grown on glass cover slips and placed in medium containing 10 μM VM26 (G to L) for 24 h and 48 h. Cells were immunostained with anti-p21 rabbit polyclonal antibody and CY3-conjugated donkey anti-rabbit secondary antibody (A, C, E, G, I, K). The nuclei were counterstained with Hoechst 33258 dye (B, D, F, H, J, L) and the cells were observed under Olympus BX50 microscope equipped with phase and epifluorescence optics and photographed using Kodak Ektachrome film.
Magnification: 650X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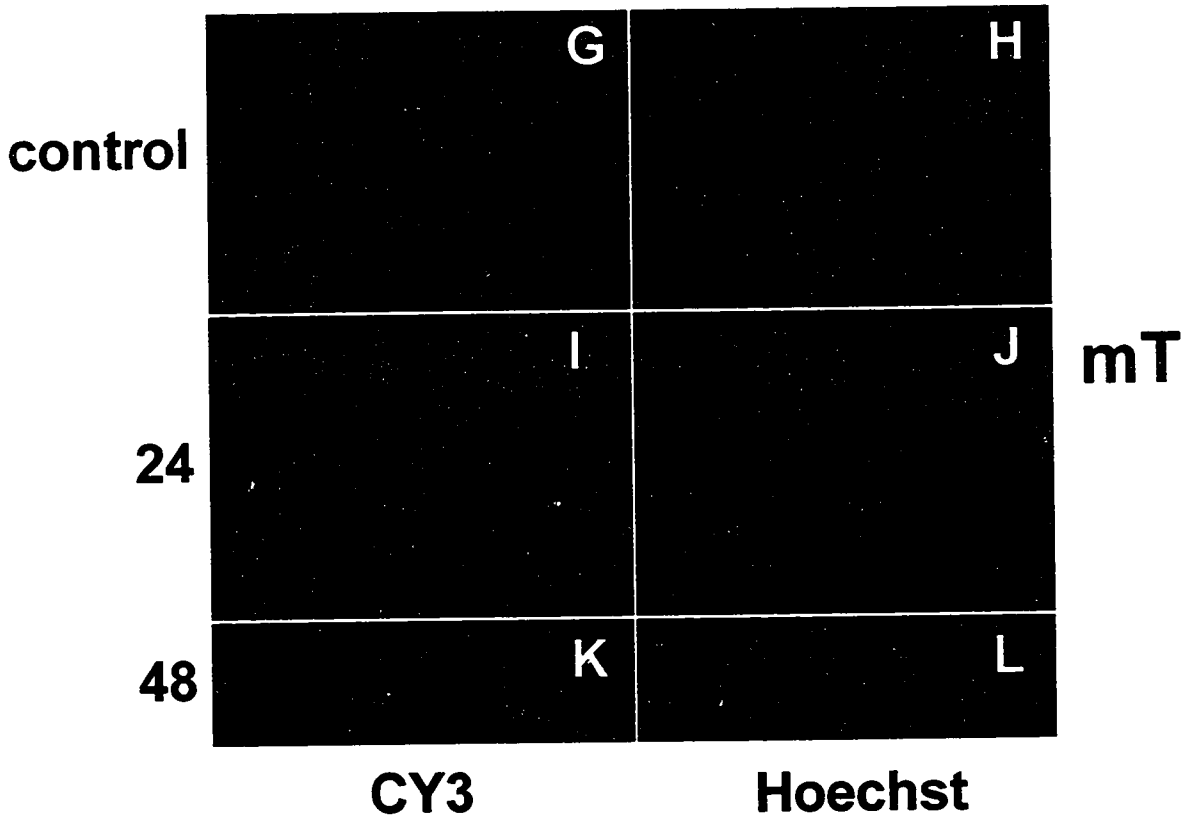
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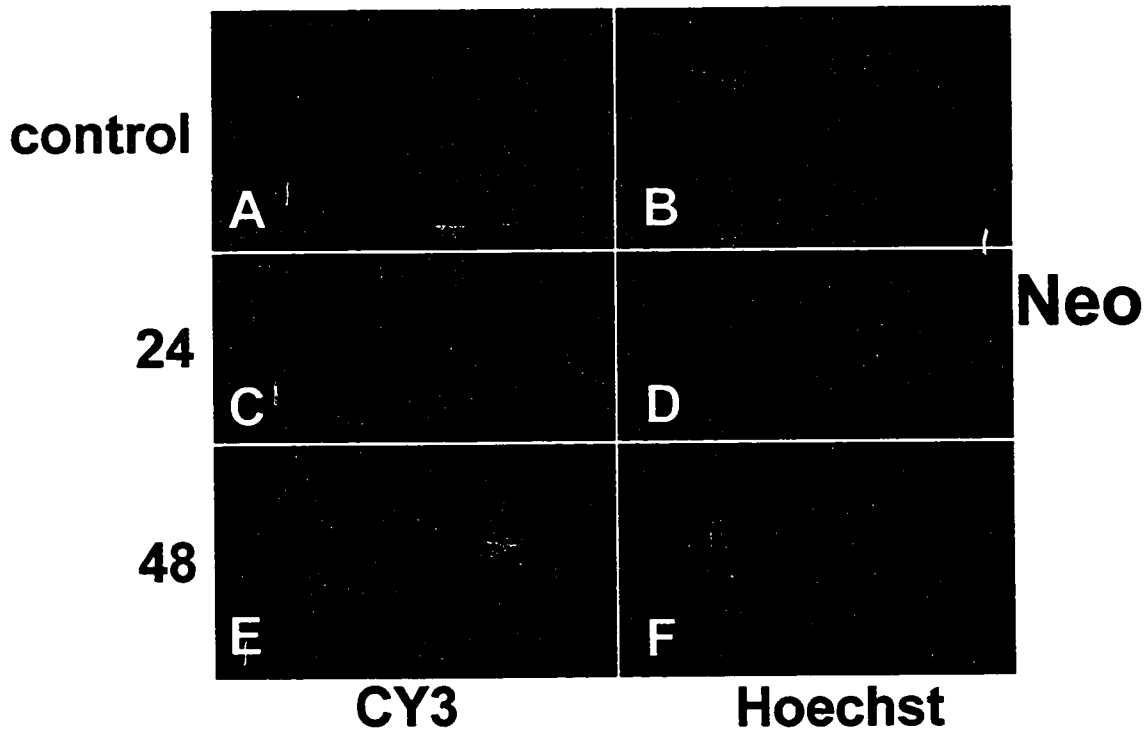
Figure 18: Changes in DNA-PK following removal of growth factors

Neo (A to F) and mT (G to L) cells were grown on glass cover slips and placed in serum-free medium for 24 h and 48 h. Cells were immunostained with anti-DNAPK1 rabbit polyclonal antibody and CY3-conjugated donkey anti-rabbit secondary antibody (A, C, E, G, I, K). The nuclei were counterstained with Hoechst 33258 dye (B, D, F, H, J, L) and the cells were observed under Olympus BX50 microscope equipped with phase and epifluorescence optics and photographed using Kodak Ektachrome film.
Magnification: 650X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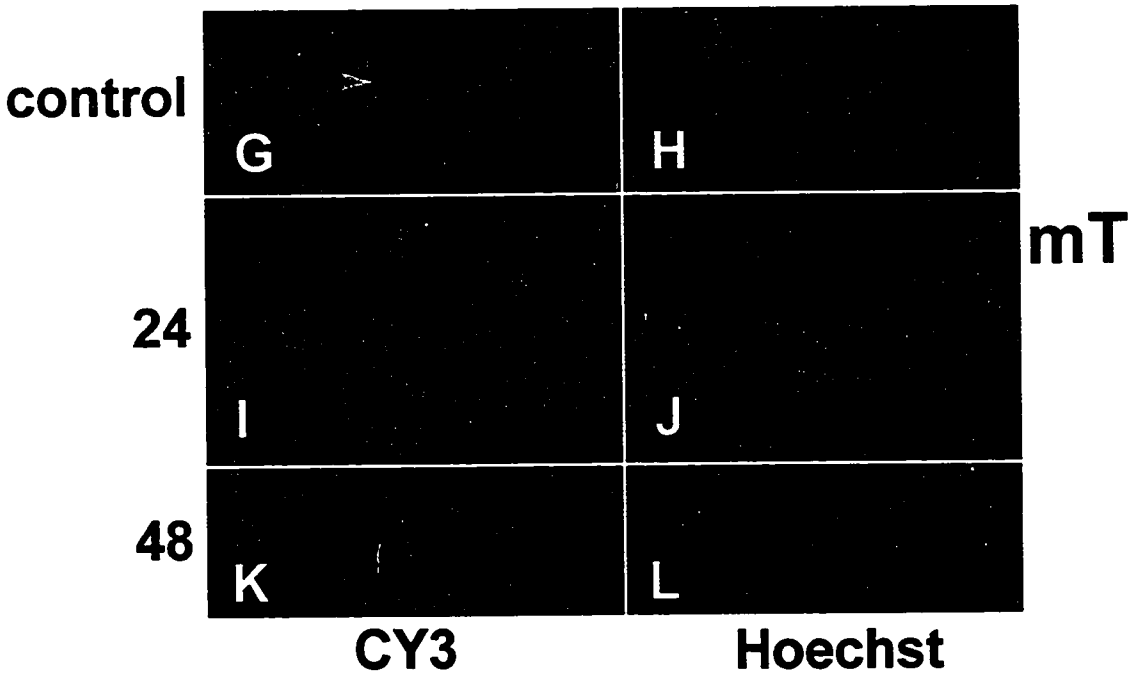
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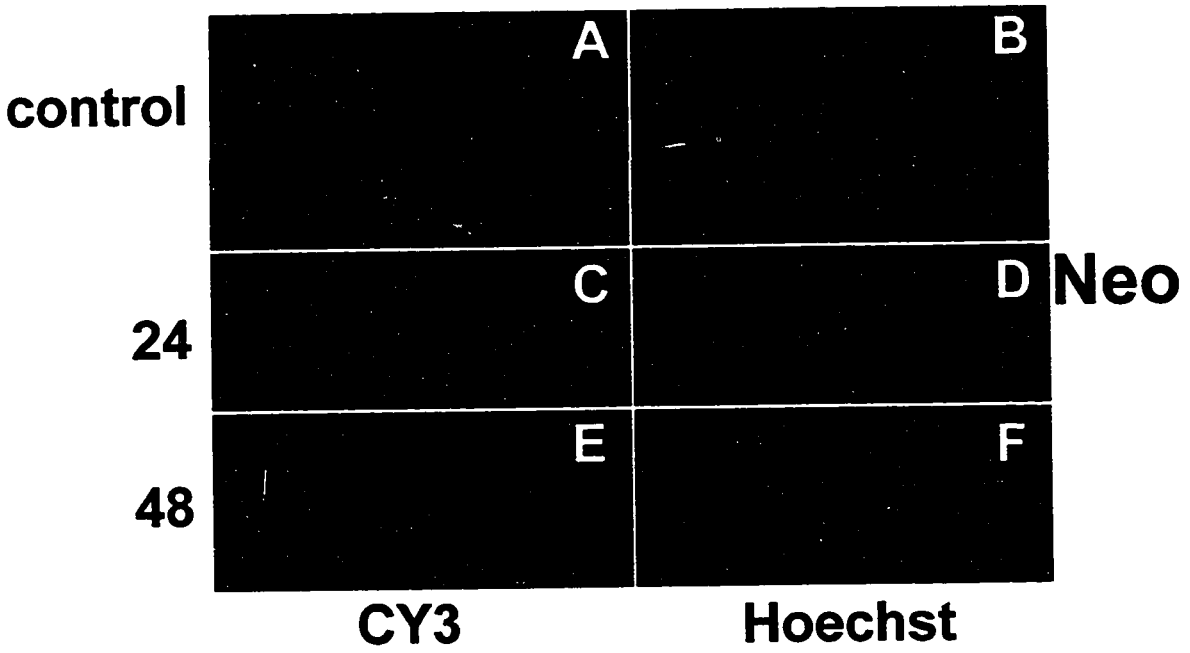
Figure 19: Changes in DNA-PK following VM26 treatment

Neo (A to F) and mT (G to L) cells were grown on glass cover slips and placed in medium containing 10 μM VM26 (G to L) for 24 h and 48 h. Cells were immunostained with anti-DNAPK1 rabbit polyclonal antibody and CY3-conjugated donkey anti-rabbit secondary antibody (A, C, E, G, I, K). The nuclei were counterstained with Hoechst 33258 dye (B, D, F, H, J, L) and the cells were observed under Olympus BX50 microscope equipped with phase and epifluorescence optics and photographed using Kodak Ektachrome film. Magnification: 650X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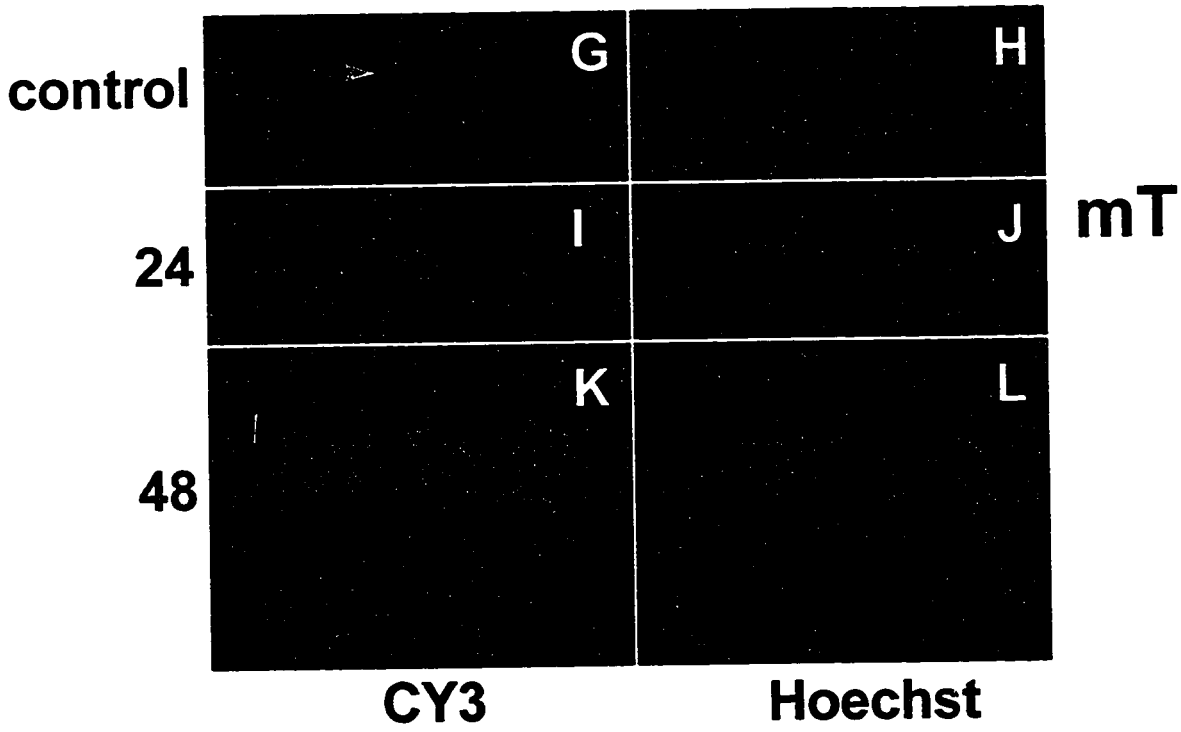
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could not be defined. No other cytoplasmic staining was detected. Within 24 hours of serum deprivation, the DNA-PK signal was completely undetectable in the resistant Neo cells (Fig. 18C). After 48 hours, the protein could be detected, but at an extremely low intensity (Fig. 18E). At this time, there was an obvious distinction between the nuclear and perinuclear staining of DNA-PK, both of which were present. Staining of DNA-PK in control mT cell showed that the signal was less intense than that seen in Neo cells but had the same distribution to nuclei and the discrete cytostructure (Fig. 18G). Upon the removal of serum, the DNA-PK signal decreased in mT cells (Fig. 18I and 18K). This did not change during the 48 hour experiment; no increase in the protein signal was detected at any time.

When VM26 was added to Neo cells, the DNA-PK signal decreased to a very low level within 24 hours (Fig. 19C). However, the signal did not disappear. Immunostaining showed that the protein expression did not increase at later (i.e. 48 hour) times in any cells that survived treatment with the drug (Fig. 19E). Immediately following the addition of VM26 to mT cells, the amount of DNA-PK decreased from control levels (Fig. 19I). Staining was very weak, similar to Neo cells in the same conditions. However, a significant increase in DNA-PK was detected in mT cells after 48 hours in the drug (Fig. 19K). The staining was extremely bright and diffuse in the nuclei, as these cells continued to survive the drug treatment.

Discussion

For a cell to become tumorigenic, it must undergo three changes i.e. it must become immortalized, acquire a neoplastic phenotype and develop metastatic potential. An immortalized cell has acquired the property of indefinite growth, usually without any other phenotypic changes. Neoplastic transformation describes the ability of a cell to overcome the normal constraints of growth and replication. Transformed cells proliferate rapidly and are unable to adopt a state of quiescence. In fact, they die rapidly if their proliferation is inhibited. (Alberts et al. 1989, Studzinski 1989, Weinberg 1985). A transformed cell must first become immortalized, then it can acquire metastatic properties. Metastasis is the feature by which a cancer cell gains the ability to invade normal tissue, so that a cancer cell can move away from the tissue of origin and establish a new colony elsewhere in the organism. This process marks the distinction between a tumor that is clinically benign and one that is invasively malignant. Any established cell line has, by definition, become immortalized. However, non-tumorigenic established cells remain subject to growth control. For example, they cannot maintain anchorage independent growth, and they are subject to serum dependence and density-dependent inhibition of growth. These features are typically used to assess cellular phenotype (Tannock and Hill 1987, Alberts et al. 1989, Phillips and Nation 1995). Using these criteria, it was established that mT antigen of the polyoma virus was able to induce the neoplastic transformation of T51B cells. Specifically, their morphology was altered, cells showed reduced adherence to the substratum and expressed a rounded shape with few adhesion plaques. They also multiplied and surpassed confluence to form foci in

the culture flask. In addition, after being plated in soft agar, they remained viable and proliferated rapidly, forming many colonies. In contrast to the mT cells, the nontransformed Neo cells were large, flat and tightly adherent to the culture flask. They exhibited strict dependence on adherence, characteristic of nontransformed cells, and died rapidly in soft agar. A search of the literature did not identify any previous studies on growth kinetics and phenotypic alterations of mT-transformed rat liver epithelial cells.

Although the polyoma virus comprises 3 tumor antigens, it is specifically the middle T antigen that is responsible for cell transformation. Neoplastic transformation by mT has been extensively studied using rat fibroblasts F2408 and FR3T3 (Gelinas and Bastin 1985, Asselin et al. 1983, Treisman et al. 1981). The mT-transformed fibroblasts were shown to grow to high saturation densities and formed foci in monolayer cultures. They also showed phenotypic changes, including changes in the appearance of the cells and the ability to grow in semi-solid medium. Transformed fibroblasts were also rounded and elongated, similar features to those seen in the mT-transformed T51B cells. In all the studies involving mT transformation of rat fibroblasts it was concluded that the presence of the mT protein alone was not sufficient to maintain growth in low serum conditions. However, this characteristic could be overcome by the presence of the N-terminus of polyoma large T antigen, which is normally required for immortalization of polyoma virus-transformed cells.

Although mT antigen can associate with cell membranes via its C-terminus, it has been shown to be predominantly cytoplasmic. In fact, immunostaining revealed the protein in the cytosol, predominantly in perinuclear regions. This distribution reflects results obtained in previous studies (Griffin and Dilworth 1983). The protein does not have any

activity itself, but it binds to specific cellular proteins with its N-terminal, harnessing their functions to cause neoplastic transformation. The best characterized of these proteins are protein phosphatase 2A (PP2A) and tyrosine kinase ($pp60^{c\text{-}src}$) (Treisman 1981, Dunant et al. 1996, Rassoulzadegan et al. 1982, Kaplan et al. 1988, Dilworth 1995, Asselin et al. 1983).

$pp60^{c\text{-}src}$ is the cellular homologue of the Rous sarcoma virus oncogene product. It is a non-receptor tyrosine kinase involved in cell signaling processes. Previously, it has been shown that mT binds directly to $pp60^{c\text{-}src}$ (Bolen et al. 1984, Courtneige 1985, Bolen and Israel 1985). Normally, phosphorylation of Tyr527 on c-src inhibits its activity. Association with mT prevents the inhibiting phosphorylation of the kinase, therefore c-src remains active. When bound to mT, c-src can phosphorylate the viral protein on Tyr315 and Tyr250. Phosphorylated on Tyr315, mT becomes a binding site for phosphatidylinositol 3'-OH kinase (PIK3), causing its activation, increased levels of DAG and, subsequently, activation of several isoforms of PKC. In addition, when Tyr250 of mT is phosphorylated, it acts as a binding site for the Shc oncoprotein. Shc is further phosphorylated by c-src and becomes activated, recruiting the guanine nucleotide exchange factor Sos, which eventually activates $p21^{ras}$ (Dilworth 1995). Stimulation of ras initiates the MAPK signalling cascade and subsequent cell transformation (Hill and Treisman 1995, Cobb et al. 1994, Davis 1995, Khosravi-Far et al. 1995). Another MAPK-associated protein, PP2A, is a major soluble serine/threonine specific phosphatase that is found in most cells. It normally functions within a cell by dephosphorylating MAPK, thus inactivating it. With its N-terminal, mT binds PP2A and inhibits its function, causing the constitutive activation of MAPK (Pallas et al. 1990).

MAPKs are a family of protein kinases whose prototype members are the mammalian extracellular signal-regulated kinases, ERK1 and ERK2. The functional forms of these proteins are normally phosphorylated on Tyr204/Thr202 and Tyr185/Thr183, respectively (Cobb et al. 1994). It is these phosphorylated residues on the kinases that were detected by the anti-aMAPK antibody used in these studies. Examination of aMAPK in mT cells revealed that the dually phosphorylated signaling proteins were constitutively present in the entire population, indicating that the viral antigen-mediated signaling events were functioning in the manner described above. An intense signal was detected not only in the cytosol, but also in the nuclei of these cells. In contrast, in Neo aMAPK was detected exclusively in mitotic cells. This is consistent with the known function of MAPK in nontumorigenic cells and the localization correlated well to the physiological targets of MAPK, which are situated in nuclei (Davis 1995).

In the absence of growth factors, mT cells died by apoptosis. Both morphological and biochemical characteristics of apoptosis were observed in the dying cells. It has been known for some time that the removal of necessary growth factors, survival factors or tissue-specific signals can induce an apoptotic pathway (Umansky 1996, Fesus et al. 1991, Williams 1991). For example, the importance of adrenocorticotropic hormone for adrenal cortex cells, neurotrophic factors for neurons and fibroblast growth factor for endothelial cells has been documented. These cells, when deprived of their respective growth factors, show typical apoptotic features (Duvall and Wyllie 1986, Edgington 1987, Raff 1992, Bursch et al. 1992, Cohen 1993, Hibner and Coutinho 1994). In general, forcing a tumorigenic cell to proliferate under restricted conditions has been shown to cause its death

by apoptosis (Wyllie 1987, Evan et al. 1995, Raff 1992). The sensitivity to growth factor deprivation was not an inherent characteristic of T51B cells. Neo cells were able to withstand growth factor deprivation, suggesting that this response may be specific to mT-mediated activities in this cell type. Examination of aMAPK in the serum-deprived cells showed that the mT cells were not capable of maintaining MAPK activity at all. The active protein became almost undetectable, indicating that the viral antigen required stimulation at the level of the cell membrane to sustain its ability to affect MAPK. No change in the aMAPK signal was seen in the Neo cells. The protein was still detected only in mitotic cells. VM26 treatment of the mT cells did not alter the functionality of MAPK. The cellular distribution of the active protein was the same as in control cells. Surprisingly, however, aMAPK was seen in many apoptotic Neo cells. This suggested that the chemotherapeutic drug was capable of interfering with MAPK inactivation in Neo cells. The serine/threonine -specific PP2A is one of the enzymes implicated in the dephosphorylation of MAPK. As described earlier, it has been shown to associate with mT and this association prevents it from dephosphorylating MAPK. However, there is another family of phosphatases involved in the inactivation of MAPK which are capable of dephosphorylating both tyrosines and threonines on aMAPK. The dual specificity MAPK kinase phosphatase-1 is one example of these. Therefore, it is conceivable that VM26 altered the function of one or more phosphatases, thereby causing MAPK to remain phosphorylated in drug-treated cells. However, this clearly did not abrogate the Neo cell apoptotic response.

MAPKs were originally described as serine/threonine kinases that could be activated by various growth factors and tumour promoters in mammalian cultured cells. It is now

recognized that these kinases are key molecules in signalling processes stimulated by growth and differentiation factors. Normally, activation of receptor tyrosine kinases by growth factors, cytokines and mitogens at the cell membrane initiates a signal cascade resulting in stimulation of MAPKKK, MAPKK and, eventually, MAPKs. These versatile enzymes are transducers for multiple intracellular signalling pathways which comprise a common route allowing signals from different growth factor receptors to converge on intracellular targets including other receptors, protein kinases, cytoskeletal proteins and transcription factors (Cobb et al. 1994, Dilworth 1995, Hill and Treisman 1995, Nishida and Gotoh 1993, Gotoh and Nishida 1995). The MAPK cascade is a complex phosphorylation network. In fact, the MAPK-mediated phosphorylation of upstream kinases is believed to play a role in positive and/or negative feedback of the cascade. MAPKs phophorylate a wide variety of targets including effector kinases and transcription factors. It has been demonstrated that aMAPK surrounds the nuclei of quiescent cells, and very little is localized in the nucleus. However, after mitogenic stimulation the aMAPK is translocated into the nucleus. It is here that it regulates the activity of a number of transcription factors (Gotoh and Nishida 1995, Davis 1995, Hill and Treisman 1995). Previous studies have indicated that the MAPK cascade is also activated by numerous oncogene products. This may be a general mechanism used by transforming agents to intercept cell cycle control mechanisms (Cobb et al. 1994, Khosravi-Far et al. 1995). In fact, it has been shown that stimulation of tyrosine-kinase associated growth factor receptors initiates cellular events similar to mT-mediated activities, including deregulated mitosis and subsequent cellular transformation (Treisman et al. 1981, Gélinas and Bastin 1985, Dilworth 1995, Kaplan et al. 1988, Priehs et al 1986). mT-stimulated

MAPK pathways and phosphorylation cascades demonstrate that the viral protein acts, at least partially, as a mitogen-activated growth factor receptor (Courtneige 1985, Carmichael et al. 1982, Dilworth 1995, Pallas et al. 1990, Bolen et al. 1984). In fact, this continuous signaling may not only be a mechanism for cellular transformation, but may also be the reason for serum dependence of mT-transfected cells. The requirement for a constant influx of growth factor signaling to the MAPK cascade proved to be detrimental to the cells in serum-deprived conditions. The Neo cells, which were not under constant stimulation to proliferate via MAPK signaling were able to tolerate serum-free conditions. In fact, they became quiescent. On the other hand, the mT cells could not withstand the lack of growth factors and started to die within 24 h, unable to support MAPK signaling under these circumstances.

The most significant observation derived from this study was the resistance of mT cells to treatment with the topoisomerase II inhibitor, VM26. In fact, these cells were able to withstand 48 hours of continuous exposure to the DNA-damaging drug without the activation of apoptosis. The cells stopped proliferating almost immediately, but they did not die until 3 days later. In contrast, the drug treatment of Neo cells resulted in their rapid death, again indicating that drug resistance was not an inherent feature of T51B cells.

VM26 is a topoisomerase II inhibitor that causes extensive DNA damage, thereby stimulating the apoptotic pathway (Umansky 1991, Hickman 1992, Sen and D'Incalci 1992). Topoisomerase II functions to relax supercoiled DNA. Normal physiological events such as replication, transcription and recombination depend on the activity of this enzyme. It accomplishes this by causing transient double strand DNA breaks and bridging the break to

allow uncoiling of DNA before rejoining the two strands. The creation of DSB is a highly controlled occurrence. VM26 takes advantage of this event to stabilize the transiently cleaved DNA-topoisomerase II intermediate, and prevent re-sealing of the genetic material. The resulting double-strand breaks usually initiate an apoptotic pathway in treated cells (Walker et al. 1991, Bertrand et al. 1991, Tsuruo and Ogawa 1992, Roy et al. 1992, Walker et al. 1993).

DNA breakdown in apoptosis produces a characteristic pattern of high and low molecular weight fragments. The fragments 50 kb and larger, referred to as the high molecular weight fragments, are thought to arise from cleavage of DNA chromatin loops, whereas the smaller fragments, resulting from internucleosomal DNA cleavage, form the classical DNA ladder pattern. Although this DNA ladder has become the hallmark of apoptosis, there is considerable variability in the extent to which different cell types fragment their DNA. In any given cell type fragmentation proceeds to a specific point, regardless of cell death stimulus, but is never complete (i.e. total degradation all the way to mononucleosomes or nucleotides) in any cell type. Thus, MCF-7 breast cancer cells produce only HMW fragments, whereas thymocyte primary cultures and many lymphocyte cell lines produce predominantly DNA ladders (Walker et al. 1995, Pandey et al. 1997, Walker et al. 1997). The T51B cells used in this study produced HMW DNA fragments, but no ladder, when they died by apoptosis. When treated with VM26, mT cells showed the initial HMW damage caused by the drug. However, the DNA degradation did not proceed further, indicating that these cells were likely capable of activating a DNA repair mechanism.

Of many proteins implicated in the induction of the apoptotic response, p53 occupies

a central role. This tumor suppressor protein participates in genome surveillance for DNA damage and in recruitment of the repair machinery. Studies have shown that DNA damage-induced p53 stabilization may either inhibit cell growth, to allow DNA repair processes, or in the case of severe damage, initiate apoptosis. DNA damaging drugs are known to cause increased stability of p53 within a few hours (Polyak et al. 1996, Fritsche et al. 1993, Cox and Lane 1995, Lane et al. 1994, Levine 1997, Kastan et al. 1991). In addition, growth factor deprivation has been shown to initiate p53 response pathways (Zhu and Russell 1993, Demers et al. 1994). As a transcription factor, p53 upregulates the expression of several other genes such as p21, GADD45 and Bax, which carry out more specific functions related to DNA damage (Smith et al. 1994, Boise et al., Korsmeyer 1995, Chin et al. 1997, Maxwell et al. 1997).

Examination of p53 in both cell lines by Northern blotting showed that the mRNA was expressed at a very low level. However, the protein could not be detected by immunofluorescence staining using a commercially available antibody. In general, the expression of p53 was lower in control mT cells than in Neo cells. After both serum removal and VM26 treatments, no significant changes in expression were observed. However, according to recently obtained data not included in the thesis, VM26 caused stabilization of p53 protein in both cell lines as detected by Western blotting. Both cell lines were examined for changes in the p53-regulated proteins which are directly implicated in DNA damage detection and apoptosis.

One of these proteins was Bax. p53 is known to be a direct transcriptional activator of Bax (Gotz and Montenarh 1995, Maxwell et al. 1997). The expression of this Bcl-2-

related protein was not consistent with its function as a determinant of apoptosis. Neither of these cell lines expressed Bcl-2 (data not shown). A role for Bax as an accelerator of apoptosis has previously been well established (Knudson and Korsmeyer 1997, Oltvai et al. 1993, Korsmeyer, 1995). While it had previously been believed that Bax and the anti-apoptotic Bcl-2 might work in concert to regulate apoptosis, it is now clear that the two proteins function independently. In fact, the ratio of Bcl-2 to Bax may represent one cell-autonomous rheostat that predetermines a cell's life or death response to an apoptotic stimuli. It is important to note that an association between Bax and Bcl-2 exists in cells even before a death-inducing stimulus is received and that Bax itself does not cause cell death without such a stimulus. For example, sympathetic neurons express high levels of Bax mRNA, yet will not undergo apoptosis unless they are deprived of growth factors. Even cells stably overexpressing Bax proliferate normally, and the protein only accelerates cell death after an external signal. Bax expression, at least at physiological levels, is not lethal to cells; mRNA is expressed in normal tissues and in a variety of cell lines prior to a death induction signal and the synthesis of Bax is not a *de novo* response that follows a death stimulus (Oltvai et al. 1993, Korsmeyer 1995, Wolter et al. 1997). In fact, there were no significant changes in Bax expression indicative of a role in the apoptotic response in either Neo or mT cells. The mRNA levels were higher in control Neo cell than in control mT cells. Serum deprivation resulted in downregulation of the message in Neo, and upregulation in mT. The Neo cells showed no change in the expression after drug treatment, but the mT cells showed induction of Bax. Clearly, in these two cell lines, the expression pattern of Bax was not consistent with

the cellular responses to apoptotic stimuli.

In mammalian cells, an important checkpoint in the G1 phase of the cell cycle is mediated by p53. This checkpoint serves a protective function by delaying S phase entry until DNA damage is repaired. Both GADD45 and p21 are thought to contribute to cell cycle arrest when DNA damage is detected (Smith and Fornace 1996, Fornace 1992). Both proteins are regulated by p53 and have been suggested to play an active role in DNA repair.

The precise mechanism of GADD45-mediated growth suppression is not known. However, it has been well documented that p21 suppresses cell growth by inhibiting Cdk function. Both proteins have been shown to interact directly with each other, as well as with PCNA, a highly conserved and essential component of the DNA repair system(Smith et al 1994, Hall et al. 1995, Gartel et al. 1997). The precise role of the interactions between the three proteins in DNA repair is not yet clear (Kelman 1997).

PCNA plays several critical roles in DNA metabolism. This 29kDa protein plays an essential role in nucleic acid metabolism as a component of the replication and repair machinery. As a component of the replication machinery, PCNA functions as the processing factor for DNA polymerase δ. There is evidence of significant stimulation of polδ activity upon PCNA binding. PCNA is also required for DNA repair, where it binds DNA polε (Kelman, 1997). The subcellular distribution of PCNA changes through the cell cycle and PCNA localizes to sites of DNA replication in S phase. PCNA levels are also elevated in response to DNA damage in vivo. It has been shown that after DNA damage occurs in the cell, there is an apparent change in the nuclear distribution of PCNA which may indicate a redistribution of the protein from sites of DNA replication to sites of DNA damage and

repair (Smith et al. 1994). In vitro repair assays have demonstrated the involvement of PCNA in DNA excision and mismatch repair processes. The mechanism by which PCNA is involved in repair remains to be elucidated, however. PCNA transcription is stimulated upon exposure to UV radiation and it has been demonstrated that this elevation of PCNA may be mediated by p53 protein. Some in vivo studies suggest a direct role for PCNA in the check-point mechanism via its interaction (direct or indirect) with other, well established check-point proteins. A possibility currently under investigation is that p21 might prevent the interaction between DNA polδ and PCNA, thus preventing replication of damaged DNA (Kelman, 1997). The GADD45 gene product binds to PCNA by its N-terminus, and GADD45 has been shown to co-immunoprecipitate with PCNA (Hall et al, 1995) (Kelman, 1997). However, the effect of GADD45 binding on PCNA remains unknown (Smith et al., 1994). The association of the two proteins, GADD45 and PCNA, is detected only after DNA damage has occurred, and the interaction between the two proteins has been shown to stimulate nucleotide excision repair (Smith et al., 1994) (Kelman, 1997). It could facilitate the formation of PCNA-containing repair complexes or it may itself associate with the DNA polymerase complex (Smith et al., 1994).

Both GADD45 and p21 proteins were detected by immunofluorescence staining in the cells' nuclei. The diffuse staining of GADD45 suggested that the protein was evenly distributed throughout the nuclei. Serum deprivation resulted in a decrease of GADD45 in both cell lines, with subsequent increase in the few mT cells that were capable of surviving for 48 h in serum-free conditions. It is possible that the mT cells were showing a delayed attempt at GADD45-mediated cell cycle arrest at this time. VM26-treatment of both cell

lines resulted in what initially seemed to be a loss of GADD45 protein after 24 h. However, bright protein staining and high expression of mRNA were detected at later times. The immunodetection of p21 revealed its localization to a few discrete areas. p21 could not be detected in serum-deprived mT cells, but the protein redistributed and was seen as very bright, diffuse staining in many of the Neo cells. This high signal in Neo cells suggested that, according to its function, p21 was initiating growth arrest, allowing the cells to survive the lack of growth factors. Redistribution of p21 protein was seen in VM26-treated Neo cells after 48 h. This delayed shift in the relocalization of p21 indicated that the Neo cells' response to drug-induced DNA damage was not immediate. By the time the cells responded, they could not repair the extensive damage that had accumulated. mT cells also showed bright, diffuse staining of p21, however the change was seen immediately within 24 h of drug treatment. This evidence suggests that mT cells initiated immediate cell cycle arrest to allow the prompt repair of DNA damage induced by VM26, and were able to prolong this activity for up to 48 h.

A significant result which provides additional evidence of an active DNA repair process in mT cells involves the DNA-dependent protein kinase (DNA-PK). DNA-PK has been implicated in DNA repair. It binds to DSB and has been suggested to allow the assembly of repair apparatus at damaged sites. Although it has been postulated to be the protein that detects DSB and reports to p53, its activation is p53-independent. This protein is a nuclear protein serine/threonine kinase that is activated by DSB. The precise function of DNA-PK in DNA DSB repair is not known. Once positioned at a DSB, DNA-PK might protect DNA ends from nuclease attack or hold them together to facilitate their religation.

In addition it could recruit other DNA repair components or activate them via phosphorylation. On the other hand, DNA-PK might remove factors, such as chromatin components or transcription factors which could otherwise interfere with DNA repair. Although no evidence for a signaling role has yet been shown, it has been postulated that the kinase may trigger phosphorylation cascades, activating cellular DNA damage signaling pathways leading to transcription apparatus, apoptotic machinery or cell cycle machinery (Jackson 1997, Lees-Miller 1996).

The level of DNA-PK decreased in both Neo and mT cells after serum removal. After treatment with VM26, its signal in Neo cells also decreased as compared to control. In contrast, the drug-treated mT cells initially showed a decrease in DNA-PK staining followed by a significant increase of the signal at later times. This change was unique to the mT cells. Although, by immunofluorescence staining criteria, the protein seemed to disappear, recent western blotting results not shown in this thesis indicated that DNA-PK was, in fact, present in the nuclei throughout the treatment. This suggested that the nuclear distribution of the protein changed following drug addition, probably due to its active role in the process of DNA repair. This could indicate that in these cells, the protein detected DNA DSB and was capable of initiating a repair response. This significant result supports other evidence indicating the mT cells' enhanced capability for growth arrest and repair of DNA damage.

The ability of mT cells to reseal DSB is not a common occurrence in tumorigenic cells. In fact, many DNA-damaging chemotherapeutic drugs induce apoptosis in this manner. However, the cytotoxic drugs used for treatments frequently generate additional

genetic mutations which assist the cell to resist death. Although some cancers are becoming increasingly easier to eliminate using antineoplastic drugs, some of the major human cancers have become chemoresistant. Intrinsic resistance and acquired resistance are the major factors which limit the successful use of chemotherapy (Phillips and Nation 1995, Hickman 1992, Grindley 1994, Bursch et al. 1992, Sen and d'Incalci 1992). Intrinsically chemoresistant tumors frequently exhibit a multidrug resistance (MDR) phenotype, which indicates their cross-resistance to several anticancer drugs. A wide range of metabolic or structural properties of cells may lead to this phenotype. For example, membrane proteins from MDR cells contain a 170 kDa P-glycoprotein, which plays a major role in drug resistance. The degree of resistance can be correlated with the amount of P-glycoprotein expressed in the cell membrane. Human P-glycoprotein is encoded by the *MDR1* gene and represents an integral plasma membrane transport protein that recognizes chemically diverse cytotoxic agents as substrates for ATP-dependent efflux. The *MDR* genes comprise a subfamily of a large superfamily known as ATP binding cassette transporters, or traffic ATPases. Depending on their physiological transport function, they are located at the cell surface or within intracellular membranes. P-glycoprotein has been shown to bind ATP directly, a prerequisite for coupling of ATP hydrolysis to the transport process. In this manner, P-glycoprotein can cause decreased uptake and increased efflux of drugs. VM26 is part of a family of chemotherapeutic drugs related to etoposide (VP-16), which has been implicated in the relation between cross-resistance of tumour cells and expression of P-glycoprotein (Tannock and Hill 1987, Fine et al. 1996, Chambers et al. 1990, Germann et al. 1995).

Two methods were applied in this study to assess cell viability, i.e. trypan blue exclusion and the MTT assay. Trypan blue was used to establish integrity of the plasma membrane. Typically it identifies cells in advanced stages of apoptosis. The MTT assay was used as an indicator of mitochondrial enzyme activities. According to this assay, serum-deprived cells, both Neo and mT, lowered their mitochondrial activity, most likely due to the abrogation of signaling from the cell surface to the organelles. The drug-treated mT cells, however, behaved quite differently. These cells showed remarkably high mitochondrial activity throughout the drug treatment. Even after 72 h, when only 20% of cells survived treatment, the MTT reading was still at 70% of control values. This ability was unique to mT-mediated transformation, since in Neo cells the drug caused a rapid drop in mitochondrial function. This highly significant result indicates that these drug-resistant mT cells may, in fact, be displaying a MDR phenotype. Their high mitochondrial activity may reflect their ability to support an ATP-dependent drug efflux mechanism characteristic of MDR cells.

Increasing evidence shows that PKC-mediated signaling pathways may be involved in the MDR phenotype (Germann et al. 1994, Kuo 1994, Fine et al. 1996, Chambers et al. 1990). In mT cells, PKC could be activated by viral antigen stimulation of DAG production. It has been shown that PKC is a modulator of MDR through a complex pattern of regulation involving the drug efflux pump. Cells selected for multidrug resistance generally contain increased levels of PKC. In fact, several laboratories have demonstrated that exposure of MDR cells to activators of PKC stimulated phosphorylation of P-glycoprotein, reduced drug accumulation and enhanced drug resistance. Conversely, treatment of MDR cells with PKC

inhibitors had the opposite effect. The interaction between mT, PKC and P-glycoprotein may provide one additional mechanism for drug resistance in mT-transformed T51B cells. This phenomenon, however, requires further exploration.

In conclusion, it was determined that middle T antigen of polyoma virus caused the neoplastic transformation of rat liver epithelial cells. This phenotype resulted from the constitutive stimulation of the MAPK pathway by the viral protein. In addition, the transformed cells acquired sensitivity to growth factor deprivation, which may also have been a consequence of aberrant MAPK signalling. The mT cells were resistant to VM26-induced apoptosis. This resistance was, at least partly, the result of p21 and GADD45-mediated growth arrest and subsequent DNA repair by DNA-PK. Furthermore, the cells might have acquired a MDR phenotype, as indicated by increased mitochondrial enzyme activities, which could potentially activate efflux of VM26 and allow DNA repair to commence.

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