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Radiation-Induced Apoptosis and the Adaptive Response in Human Lymphocytes

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ABSTRACT

Apoptosis is a genetically programmed cell death process which can be activated in cells by a variety of toxic agents including ionizing radiation. It has generally been assumed that radiation-induced DNA damage is responsible for activating the apoptotic process; however, some recent data suggests that oxidative damage of the plasma membrane may be more important. In the present study we have used DNA and membrane active radiomimetic agents to investigate the role of DNA damage and membrane oxidative damage in radiation-induced apoptosis in human lymphocytes. Our results demonstrate that these two classes of agents trigger apoptosis through distinct pathways. The lipophilic oxidizing agents, t-butyl hydroperoxide and cumene hydroperoxide, triggered apoptosis through a rapid induction pathway (within 2 hours) which did not require active protein synthesis. In contrast, ionizing radiation and the chemical DNA damaging agents bleomycin, cisplatin, and m-amsacrine triggered apoptosis through a slow kinetic pathway (peaking at 48-72 hours) which was dependent on active protein synthesis. Further studies demonstrated that radiation-induced apoptosis did not correlate with membrane oxidative damage. Radiation was found to be much less effective (> 10-fold) than t-butyl hydroperoxide at stimulating lipid peroxidation at doses at which these two agents produced similar levels of apoptotic cell death. Furthermore, the lipophilic antioxidant vitamin E did not protect against radiation-induced apoptosis at concentrations at which it significantly reduced t-butyl hydroperoxide induced lipid peroxidation and subsequent apoptotic cell death. On the other hand, radiation-induced apoptosis was shown to correlate with DNA damage. The DNA repair inhibitor cytosine arabinoside (Ara-C) was shown to inhibit the repair of radiation-induced DNA strand breaks and to significantly enhance the level of subsequent radiation-induced apoptotic cell death. Furthermore, it was shown that while peroxide-induced DNA single-strand breaks are rapidly repaired and ineffective at triggering apoptosis, inhibiting the repair of these lesions using Ara-C results in a significant induction of apoptotic cell death. These results suggest that radiation-

induced apoptosis in lymphocytes is triggered by a signal related to slowly repaired (or unrepairable) DNA lesions.

It has previously been reported that pre-exposure of mitogen-stimulated lymphocytes to a low dose of ionizing radiation can reduce the extent of chromosomal damage induced by a subsequent high dose radiation exposure. This phenomena has been termed an "adaptive response" to ionizing radiation and has been proposed to involve the induction of a DNA repair process by the low dose radiation exposure. In the present study we investigated the possibility that pre-exposure of lymphocytes to a low dose of ionizing radiation could modify the extent of apoptosis induced by a subsequent radiation exposure. Our results demonstrate that when lymphocytes from various donors were pre-exposed to a 0.1-Gy (adapting) dose of ionizing radiation the extent of apoptosis induced by a subsequent 2-Gy (challenge) dose of radiation was either significantly enhanced (mean increase of 26.1 $\pm$ 6.8%) or did not change. This sensitization effect was shown to be significantly greater when the adapting exposure was given 6 hours prior to the challenge exposure than when it was given immediately before suggesting that the sensitization effect involves an induced cellular response and does not simply result from an additivity of the lesions produced by the two radiation exposures. Furthermore, we show that this increased sensitivity to radiation-induced apoptosis could be triggered in cells pre-exposed to either a low dose of ionizing radiation or a low concentration of t-butyl hydroperoxide. On the other hand, these pretreatments did not affect the level of apoptosis induced by exposure to a high concentration of the membrane oxidizing agent t-butyl hydroperoxide. Since an increase in the elimination of genetically damaged cells by apoptosis could reduce the risk of cancer from exposure to radiation or other DNA damaging agents, this cellular sensitization for apoptosis may represent a novel adaptive response mechanism.

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List of Abbreviations

Ara-C	cytosine arabinoside
BCNU	1,3-bis(2-chloroethyl)-1-nitrosurea
bFGF	basic fibroblast growth factor
CDTA	trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid
cGy	centiGray (SI unit for absorbed radiation dose)
CHX	cycloheximide
cPNA	cis-parinaric acid
Dex	dexamethasone
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA-PK	deoxyribonucleic acid - protein kinase
EDTA	ethylenediaminetetraacetic acid
FADU	fluorescence analysis of DNA unwinding
FIGE	field inversion gel electrophoresis
Gy	Gray (SI unit for absorbed radiation dose)
h	hours
HMW	high molecular weight
hprt	hypoxanthine phospho-ribosyltransferase
IURD	Iododeoxyuridine
kbp	kilobase pairs
LMDS	locally multiply damaged site
LMP	low melting point
LOOH	lipid hydroperoxide
m-AMSA	m-amsacrine
MEF	mouse embryo fibroblast
MMS	methyl methane sulphonate
PBS	phosphate buffered saline
PFGE	pulsed field gel electrophoresis
PKC	protein kinase-C
PUFA	polyunstaurated fatty acid
scid	severe combined immunodeficiency
SDS	sodium dodecyl sulfate
t-BuOOH	tertiary butyl-hydroperoxide
TdT	terminal deoxynucleotidyl transferase
TPA	phorbol 12-myristate 13-acetate
Tris	tris(hydroxymethyl)aminomethane
UVC	ultraviolet-C radiation

GENERAL INTRODUCTION

Ionizing radiation has become an integral part of modern technology. The exploitation of atomic radiation in medicine, in industry, and in power production has steadily increased over the years and has proven to be of tremendous benefit to society. However, this increased use of radiation has been accompanied by a growing concern over the potential detrimental effects of radiation exposure - the most prominent concern being the risk of radiation-induced carcinogenesis. While there is no doubt that high doses of radiation can be harmful to living organisms, the effects of low doses remains highly controversial. To accurately assess the risks of low dose radiation exposure we must fully understand the existing biological defence systems as well as the ability of such systems to adapt to the environment including their radiation environment.

Cellular Damage Induced by Ionizing Radiation

Radiation Chemistry

Ionizing radiation is typically classified as being either *particulate* or *electromagnetic* in nature. Particulate types of radiation include extremely high energy particles such as electrons (β-rays), neutrons, protons, and small nuclei (e.g. α-particles); whereas, electromagnetic types of radiation such as X-rays or γ-rays consist of high energy photons. The deposition of energy in cells by

radiation can lead to excitation of electrons in the outer orbitals of biological molecules. In the case of ionizing radiation the amount of energy deposited is sufficient to cause ejection of these electrons (ie. cause ionization) resulting in the breaking of chemical bonds and the production of reactive free radicals.

When cells are exposed to ionizing radiation, damage is produced in particular types of biological molecules roughly in proportion to their relative mass within the cell. Radiation-induced damage to these molecules can occur by either a *direct* or *indirect* action depending on the site of the initial energy deposition events (Figure 1). The damage is considered to be direct when the radiation energy is directly absorbed by the target molecule. Alternatively, radiation can interact with other cellular molecules producing free radicals that are able to diffuse within the cell and interact with the particular target molecule. This is referred to as indirect radiation damage. Since cells consist of approximately 80% water, indirect damage of biological molecules results primarily from their interaction with free radicals produced by radiolysis of water. The most potent of these is the highly reactive hydroxyl radical which can add or strip a hydrogen atom from an organic molecule, thereby altering its chemical structure. Studies using high concentrations of agents like dimethyl sulfoxide to scavenge these diffusable free radicals have demonstrated that approximately two-thirds of the damage to biological macromolecules results from the indirect action of radiation (Reuvers *et al.* 1973).

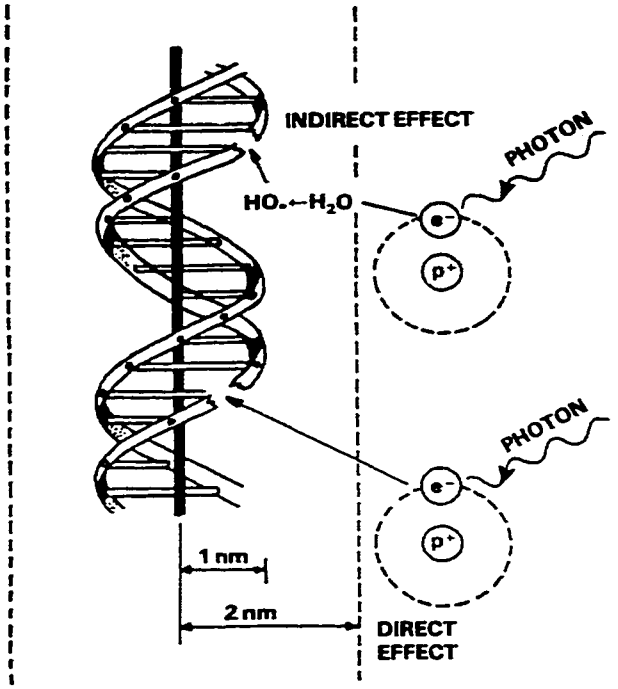

Figure 1. Direct and Indirect Actions of Ionizing Radiation. The direct and indirect actions of ionizing radiation on a molecule of DNA is shown, although similar reactions are expected to occur in other major cellular macromolecules (Tubiana *et al.* 1990).
Direct Effect: an ionizing photon interacts directly with an electron in the DNA molecule itself.
Indirect Effect: an ionizing photon interacts with a water molecule to produce a reactive free radical (e.g. HO•) which can then interact with the DNA molecule.

Fortunately, cells are equipped with a variety of endogenous free radical scavengers such as glutathione, ascorbate (vitamin C), and α-tocopherol (vitamin E) as well as antioxidant enzymes like superoxide dismutase, catalase, and glutathione peroxidases which help protect them against free radical mediated damage. These antioxidant systems, however, are not perfect and during periods of oxidative stress, such as that which occurs during exposure to

ionizing radiation, damage to major biological molecules can occur. This includes damage to structural and enzymatic proteins, cellular membranes, and most critically DNA.

Radiation-Induced Protein Damage

Free radicals generated by ionizing radiation can lead to a number of different protein modifications including oxidation, decarboxylation, and deamination of amino acids as well as larger scale structural damages such as polypeptide chain scission and crosslinking (Wolff and Dean 1986, Davies 1987, Stadtman *et al.* 1993). This damage appears to be most prominent within hydrophilic regions of proteins, likely due to the increased interaction of these moieties with hydroxyl radicals produced during radiolysis of water. Furthermore, even within these regions certain amino acid residues tend to be more susceptible than others to oxidative damage. It is thought that electrons are transferred between residues on the polypeptide chain and that certain amino acids, such as those containing sulphydryl or aromatic groups, can act as electron sinks because of their greater free radical stability (Wilks *et al.* 1992). The binding of metal ions to the surface of proteins (as well as all other biomolecules) can also contribute to damage site specificity (Chevion 1988). Transition metal ions such as iron or copper can promote the production of free radicals from oxidants like hydrogen peroxide which is known to be produced as a byproduct of water radiolysis.

It is generally thought that following exposure to radiation cells replace damaged proteins primarily through *de novo* protein synthesis as opposed to

repairing the damaged proteins themselves. In fact, cellular proteins are known to be turned over continuously as a normal part of cellular physiology and many lines of evidence suggest that damaged proteins are preferentially targeted for degradation (Davies *et al.* 1987, Stadtman 1992).

Radiation-Induced Membrane Damage

Unsaturated fatty acids within membrane structures are known to be highly sensitive to free radical mediated damage (Wagner *et al.* 1994). The high sensitivity of these biomolecules is thought to result from the ability of lipophilic free radicals to initiate a chain reaction of lipid peroxidation (*reviewed in* Girotti 1985, Gardner 1989). The chemical process of lipid peroxidation (Figure 2) is initiated by a highly energetic one-electron oxidant, such as a hydroxyl radical, which abstracts a hydrogen atom from the carbon chain of an unsaturated fatty acid resulting in the production of a carbonyl radical (L•). This lipid radical can rapidly react with oxygen to produce a lipid peroxyl radical (LOO•). Peroxyl radicals are themselves extremely reactive and can abstract a hydrogen atom from an adjacent fatty acid, resulting in the formation of a lipid hydroperoxide (LOOH) in the first fatty acid, and a lipid carbonyl radical (L•) in the second. Lipid peroxidation can thus propagate as an autolytic chain reaction whereby a single initiating free radical can result in the peroxidation of a large number of unsaturated fatty acids.

It has been shown in studies using synthetic membranes that lipid peroxidation can be initiated by free radicals produced during irradiation (Raleigh 1987). Other studies demonstrated that the oxidation sensitivity of such model membranes increased as a function of their unsaturated fatty acid content with

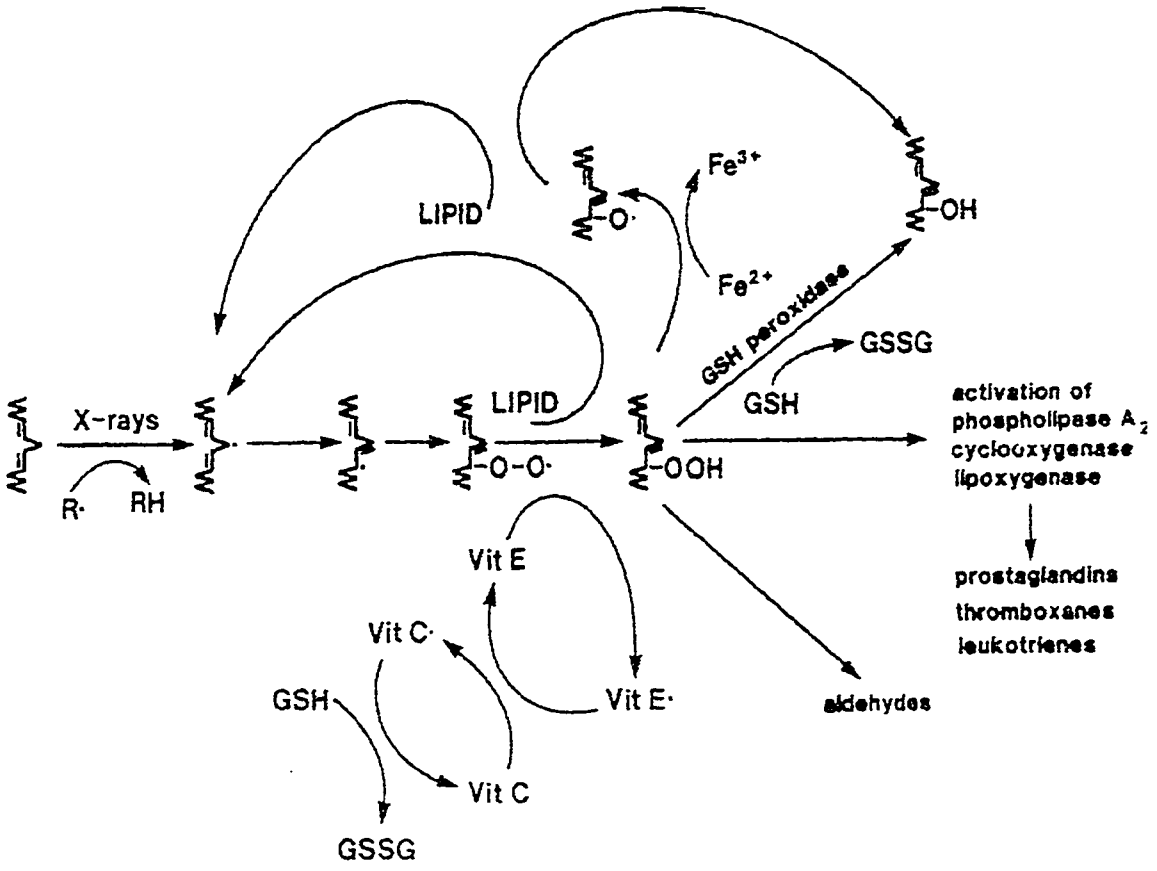

Figure 2. Radiation-Induced Lipid Peroxidation. Primary or secondary radical reactions can result in radical formation at the allylic positions of unsaturated fatty acids. Following rearrangement (diene conjugation), oxygen reacts to form a peroxyl radical. Peroxyl radicals attack other lipids to amplify damage. Hydroperoxides can decompose to electrophilic products (aldehydes) which can damage DNA, or to alkoxy radicals which can attack other lipids. Enzyme activation by hydroperoxides can produce biologically active compounds (Bump and Brown 1990).

the polyunsaturated fatty acids (PUFA) arachidonic acid and linoleic acid being the most oxidation sensitive of the naturally occurring lipid molecules (Mooibrock and Konings 1980). However, the PUFAs built into cellular membranes have proven to be considerably less radiosensitive than those in micellar or liposomal model membranes (Wolters and Konings 1982). This has been attributed to the highly efficient antioxidant and free radical scavenging activity of cells (Konings and Drijver 1979, Yamaoka *et al.* 1994). These antioxidant systems can function on two fronts. First, they can function as preventative antioxidants by scavenging or inactivating diffusable free radicals, such as hydroxyl or superoxide radicals, before they are able to interact with lipid molecules. Alternatively, antioxidants can serve a chain breaking function by reducing lipid peroxyl radicals and thereby preventing the propagation of lipid peroxidation chain reactions. The lipid soluble antioxidant vitamin E (α-tocopherol) is a critical component of this second line of defence. The vitamin E molecule consists of a phenol "head" group which is responsible for its antioxidant activity and a hydrophobic "tail" which anchors the molecule within the lipid bilayer of cell membranes (Burton and Ingold 1986, van Acker *et al.* 1993). The phenol group of vitamin E reduces lipid peroxyl radicals to lipid hydroperoxides and is itself converted to a phenoxyl radical (ie. tocopheroxyl radical) in the process (Sies and Murphy 1991). The tocopheroxyl radical is resonance stabilized and is not capable of continuing the lipid peroxidation chain reaction (Buettner *et al.* 1993). Instead the tocopheroxyl radical is believed to migrate toward the lipid bilayer surface where tocopherol is regenerated by the water soluble reducing agent vitamin C (ascorbate) (Packer

et al. 1979, Frei *et al.* 1988). The oxidized vitamin C is, in turn, reduced by enzyme systems that ultimately use NADH or NADPH as sources of reducing equivalents such as glutathione peroxidases. The potentially dangerous lipid hydroperoxides remaining in the lipid bilayer can be cleaved by phospholipase A_2, allowing glutathione peroxidase to detoxify the hydroperoxide to a fatty acid alcohol or it may be directly converted to an alcohol by phospholipid glutathione peroxidase (Thomas *et al.* 1990).

In situations of high oxidative stress, however, these defence systems can become saturated and oxidative membrane damage can occur resulting in alterations of membrane function by a number of mechanisms (*reviewed in* Koteles 1982, Girotti 1985). For example, clusters of lipid hydroperoxides produced during this process can result in regional alterations in membrane permeability allowing ion fluxes to occur across cell membranes. Secondly, lipid peroxidation reactions can affect membrane-associated proteins resulting in inactivation of membrane-associated enzymes and cell surface receptors. Finally aldehydes produced during lipid hydroperoxide decomposition can diffuse within the cell and cause damage to other critical biological molecules such as DNA.

Radiation-Induced DNA Damage

Ionizing radiation can interact with DNA either directly or indirectly to produce a number of different types of lesions including strand breaks, base alterations, and to a lesser extent DNA-DNA or DNA-protein crosslinks (Ward 1988). The amount of energy deposited by a photon of ionizing radiation is

sufficient to produce several localized ionizations (Mozumder and Magee 1966). The consequence of this pattern of energy deposition is that a significant fraction of the ionizing events cause DNA lesions in which both strands of DNA are damaged within a short segment of the molecule (Ward 1988). These lesions are called double stranded lesions and can consist of a combination of strand breaks and base alterations. Double stranded lesions are produced much less frequently than single stranded lesions and, for example, for a given dose of X- or γ-rays the yield of DNA double strand breaks has been calculated to be approximately 20-40 fold lower than that of single strand breaks (Ward 1988, Goodhead and Nikjoo 1989).

Unlike other cellular macromolecules such as proteins and lipids which are present in thousands to millions of copies per cell, each molecule of DNA is present in only one or two copies. Moreover, the instructions for the cell to grow, divide, and ultimately to produce all other cellular molecules are encoded within the sequence of the DNA. Consequently, cells posses highly efficient enzymatic repair processes which are capable of recognizing and accurately repairing a variety of different types of DNA lesions including those produced by ionizing radiation (reviewed in Sancar and Sancar 1988, Friedberg *et al.* 1996). Single stranded lesions such as strand breaks and base alterations are known to be repaired by an excision repair process in which a segment of DNA encompassing the damaged site is excised and then resynthesized using the intact complementary DNA strand as a template (Frankenberg-Schwager 1989, Hoeijmakers and Bootsma 1993, Sancar 1994), (Figure 3). Double-stranded

lesions, on the other hand, are not repaired by direct excision repair since excision of the damage on one strand of DNA would uncover the lesion on the opposing strand. This would then prevent proper resynthesis from occurring or, at least, require an error-prone resynthesis mechanism. Instead, double stranded lesions are repaired by either homologous recombination or nonhomologous end-joining. In homologous recombination damaged DNA is repaired using complementary base pairing provided by either a sister chromatid or homologous chromosome (Resnick 1976, Szostak *et al.* 1983, Friedberg *et al.* 1996). The majority of double-strand breaks in mammalian cells, however, are repaired by non-homologous end-joining in which broken ends of DNA are ligated through a process mediated by Ku-protein and DNA-PKcs (*reviewed in* Jeggo 1998, Featherstone and Jackson 1999). These repair processes and consequently the repair of double stranded DNA lesions is considered to be much slower and less efficient than the excision repair mediated removal of single stranded DNA lesions (Frankenberg-Swager 1989).

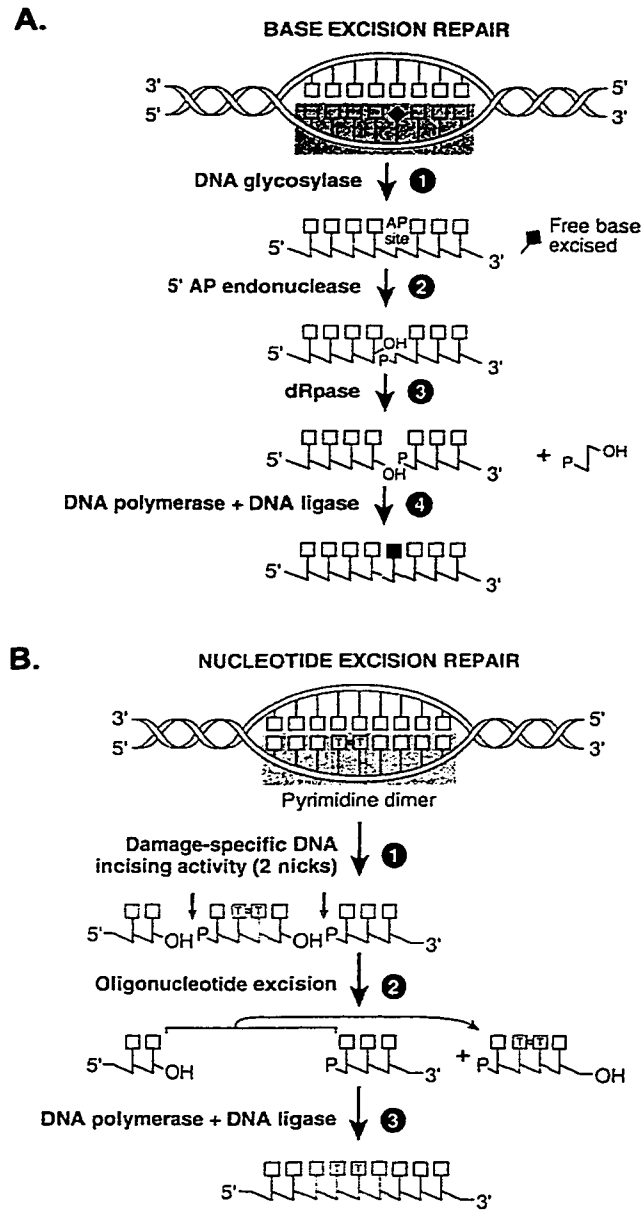

Figure 3. Model of Base and Nucleotide Excision Repair Processes [Friedberg et al. 1995]

Radiation-Induced Cell Killing

Ionizing radiation is a highly efficient cytotoxic agent. It has been estimated that only one out of every 10^6 molecules of an amino acid, phospholipid, or DNA base would be ionized at a mean lethal dose of radiation (Bump and Brown 1990). In contrast, many other oxidizing agents need to produce about a 100 times more damage to produce equal cytotoxicity (Ward 1987). Thus, radiation-induced cell killing at therapeutic doses of radiation cannot be explained in terms of massive damage to biological molecules, nor in terms of overwhelming oxidative stress.

A number of studies have investigated the cellular target(s) involved in radiation-induced cell killing. In an early hypothesis it was proposed that cell killing resulted from radiation-induced oxidative damage of cellular membranes (Alper 1977). However, subsequent studies demonstrated a lack of correlation between these two processes. For example, it was shown that radiosensitivity was not affected when the PUFA content of cell membranes was increased, even though membrane permeability was significantly increased in these cells and this endpoint was sensitive to glutathione depletion or vitamin E supplementation (*reviewed in* Konings 1987). In fact, there is now substantial evidence indicating that DNA is the primary cellular target for radiation-induced cell killing (*reviewed in* Hall 1988). The most convincing evidence comes from studies which demonstrate that radiolabelled compounds that selectively irradiate the nucleus kill cells 100-200 times more effectively than those which

selectively irradiate the cytoplasm or cell membrane (Munro 1970, Warters 1977). Even still, most of the DNA damage produced by ionizing radiation consists of isolated lesions and is considered to be irrelevant with respect to cell killing (Ward 1987). Instead, the high efficiency with which radiation kills cells is thought to lie in its ability to produce clusters of ionizing events resulting in the production of locally multiply damaged sites (LMDS) and consequently double stranded DNA lesions (Ward 1988). In support of this, agents such as hydrogen peroxide which produce single stranded lesions similar to ionizing radiation but no double stranded lesions have been shown to be ineffective at cell killing even at concentrations at which they produce 500 times as many single strand breaks as a lethal dose of ionizing radiation (Ward 1985). In fact, a strong correlation has been established between radiation-induced cell killing and the presence of unrepaired DNA double strand breaks (Joshi *et al.* 1982, Radford 1985, Radford 1986, Iliakis 1991, Obe *et al.* 1992).

Radiation is thought to induce two distinct modes of cell death depending on the cell type and radiation dose - necrosis and apoptosis (*reviewed in* Szumiel 1994). Radiation-induced cell killing has traditionally been measured by a clonogenic assay in which survival is based on a cells reproductive capacity and therefore does not distinguish between these different modes of cell death. It is generally believed, however, that necrosis is the predominant mode of cell death underlying the loss of clonogenic survival – although, this has not been clearly demonstrated. Therefore, although DNA is regarded as the primary target in

radiation-induced clonogenic cell killing the critical target(s) for radiation-induced apoptotic cell death remain unclear.

Radiation-Induced Apoptotic Cell Death

Characteristics of Apoptotic Cell Death

Apoptosis is a mode of cell death in which the cell actively participates in its own destruction. In a process that typically involves active gene expression, the cell initiates a well coordinated degradative program that results in characteristic biochemical and morphological cellular changes (*reviewed in* Kerr *et al.* 1972, Wyllie 1992, Earnshaw 1995). The most prominent changes occur within the nucleus where the sequential activation of specific proteases and endonucleases result in the collapse of chromatin structure and cleavage of DNA into distinctly sized fragments. This is accompanied by a reduction in nuclear and cellular volume (pyknosis) and the eventual formation of dense, membrane bound apoptotic bodies. Cellular membranes and organelle function remain intact through much of the death process and apoptotic cells are eventually removed, *in vivo*, by phagocytic cells without eliciting an inflammatory response (Duvall *et al.* 1985). Apoptosis differs substantially from the other principle mode of cell death known as necrosis (Searle *et al.* 1982, Wyllie 1992). Necrotic cell death is a passive process which involves the loss of membrane integrity and organelle function at an early stage resulting in swelling and lysis of the cell. This is typically followed by a marked inflammatory response resulting in further tissue damage. During necrosis the release of lysosomal enzymes associated with

membrane dysfunction can result in some DNA degradation. However, this DNA degradation is not extensive (if present at all) and yields randomly sized DNA fragments making it readily distinguishable from the DNA fragmentation which occurs during apoptosis (Searle *et al.* 1982, Duke *et al.* 1983)

The cleavage of DNA into oligonucleosomal sized DNA fragments evident as a DNA ladder on conventional agarose gels has long been considered a hallmark of apoptotic cell death (Wyllie 1980, Compton 1992). More recently, however, it has become evident that the cleavage of DNA at internucleosomal sites is preceded by its cleavage into distinct high molecular weight fragments of 50 to 300 kilobases (Walker *et al.* 1991, Brown *et al.* 1993). Furthermore, recent evidence indicates that this high molecular weight DNA fragmentation is sufficient to ensure (apoptotic) cell death and that internucleosomal DNA fragmentation may be a late and dispensable event in the apoptotic process (Collins, R. *et al.* 1992, Oberhammer *et al.* 1993, Cain *et al.* 1994, Cohen *et al.* 1994, Walker *et al.* 1994).

Role of Apoptosis in Physiological and Pathological Processes

Under normal physiological conditions apoptosis plays an integral role in the maintenance of homeostasis in essentially all tissues of the body. It is an essential mechanism involved in embryonic development, metamorphosis, regulation of tissue size and shape, and in the death of cells during normal tissue turnover (Arends and Wyllie 1991, Raff 1992). Apoptosis also functions extensively within the immune system where it is responsible for the removal of

potentially autoreactive clones during lymphocyte maturation as well as in the abolishment of immune reactions (Cohen *et al.* 1992). It has also been implicated as the mode of cell death involved in cell-mediated immune killing of pathogen-infected cells (Berke 1995). The importance of apoptosis in the maintenance of tissue homeostasis has been further emphasized by the finding that disturbances in apoptosis can contribute to a variety of pathological conditions (*reviewed in* Thompson 1995). It has been identified as a contributing factor in autoimmune diseases, AIDS, neurodegenerative disorders, and ischemic tissue damage associated with stroke or cardiac infarcts. Furthermore, downregulation of the apoptotic process has been implicated in the development and progression of certain forms of cancer (*reviewed in* Wyllie 1992).

In addition to its induction in response to various physiological stimuli, apoptotic cell death can also be triggered by variety of exogenous agents including ionophores, chemotherapeutic drugs, cytoskeletal poisons, oxidizing agents, and ionizing radiation (*reviewed in* Wertz and Hanley 1996). The ability of agents of such diverse cellular action to trigger this process suggests that apoptosis can be triggered by a number of different initiating signals which converge to activate a common cell death machinery. However, sensitivity to specific apoptotic stimuli is known to vary significantly among different cell types suggesting that not all initiating pathways are equally responsive in all cell types.

Sensitivity of Different Cell Types to Radiation-Induced Apoptosis

Human and rodent lymphoid and myeloid cells have been the most frequently used models in the study of radiation-induced apoptosis. This is because a large fraction of these cells undergo apoptosis rapidly (within 24 hours) after exposure to relatively low doses of ionizing radiation. As such, a number of different cell types derived from the hematopoietic system have been identified as being sensitive to radiation-induced apoptosis including murine thymocytes (Umansky *et al.* 1981, Yamada and Ohyama 1988, Sellins and Cohen 1987), splenocytes (Zhivotovsky *et al.* 1993), bone marrow cells (Lotem and Sachs 1993), human peripheral blood lymphocytes (Payne *et al.* 1992, Delic *et al.* 1993, Cregan *et al.* 1994, Hertveldt *et al.* 1997, Vral *et al.* 1998), and a number of different cell lines of lymphoid or myeloid origin (Olive *et al.* 1993, Radford *et al.* 1994). In addition to the sensitivity of hematopoietic cells, radiation has also been reported to trigger significant levels of apoptosis in other cellular systems including vascular endothelial cells (Fuks *et al.* 1994), spermatogonia (Hasegawa et al. 1998), intestinal crypt cells (Potten 1992, Merritt *et al.* 1993), as well as cerebellar granule (Inouye *et al.* 1992, Fritsch *et al.* 1994, Wood and Youle 1995) and hippocampal (Johnson *et al.* 1998) neurons. However, not all cell types undergo apoptosis following exposure to ionizing radiation. Cell types reported to be resistant to radiation-induced apoptosis include hepatocytes (Midgely *et al.* 1995), Chinese hamster V79 cells (Radford 1994), CHO cells (Olive *et al.* 1993), mouse L929 cells (Abend *et al.* 1995), mouse embryo

fibroblasts (Slichenmeyer *et al.* 1993), and human skin fibroblasts (Yanagihara *et al.* 1995).

In addition to normal cells, radiation-induced apoptosis has also been demonstrated in a number of tumour or tumour-derived cell lines. For example, radiation-induced apoptosis has been observed in a variety of cell lines established from human or mouse lymphomas and leukemias (Radford 1991, Baxter and Lavin 1992, Macklis *et al.* 1992, Akagi *et al.* 1993, Manome *et al.* 1993, Meyn *et al.* 1993, Shinohara and Nakano 1993, Story *et al.* 1994). Furthermore, a number of nonlymphoid tumour cells have also been reported to undergo apoptosis following exposure to ionizing radiation including colorectal adenoma and carcinoma cells (Bracey *et al.* 1995), P815 mouse mastocytoma cells (Collins, R. *et al.* 1992), DU-145 human colon carcinoma cells (Oberhammer *et al.* 1993), F9 murine teratocarcinoma cells (Langley *et al.* 1994), murine ovarian carcinoma cells (Stephens et al. 1993), human gastric carcinoma cells (Yanagihara *et al.* 1995), and some human sarcoma cells (Hass-Kogan *et al.* 1995, Soldatenkov *et al.* 1995).

Genetic Regulation of Radiation-Induced Apoptosis

BCL-2. Bcl-2 is a member of a family of related genes known to be involved in the regulation of apoptotic cell death (*reviewed in* Knudson and Korsmeyer 1997, Adams and Cory 1998). The family is known to consist of both proapoptotic members (e.g. Bax, Bad) and antiapoptotic members (e.g. Bcl-X_L, Bcl-2) and it is thought that the relative levels of expression of these opposing elements

determines the sensitivity of cells to apoptotic stimuli (Oltvai *et al.* 1993, Oltvai and Korsmeyer 1994, Chresta *et al.* 1996). In support of this model it has been shown in a number of cell systems that enforced expression of Bcl-2 or Bcl-X_L can render cells resistant to a variety of apoptotic stimuli including exposure to ionizing radiation (Sentmen *et al.* 1991, Miyashita and Reed 1992, Strasser *et al.* 1994). Furthermore, it has been reported that overexpression of Bax can enhance the sensitivity of cells to ionizing radiation as well as other DNA damaging agents (Zhan *et al.* 1994, Sakakura *et al.* 1996).

P53. The tumour suppressor protein p53 has been implicated as a key regulator of the cellular response to radiation-induced DNA damage (reviewed in Lane 1993, Ko and Prives 1996). Activation of p53 is known to trigger two distinct cellular processes - growth arrest and apoptosis. Which of these two responses prevails appears to depend on the type of cell involved as well as the existing growth conditions (Canman *et al.* 1995, Midgely *et al.* 1995). Although it has been well established that p53 mediated growth arrest proceeds through the transcriptional activation of the cyclin-dependent kinase inhibitor p21 (Di Leonardo *et al.* 1994, Dulic *et al.* 1994, Brugaralos *et al.* 1995), the mechanism by which p53 leads to activation of apoptosis has not been clearly defined. In certain cases it appears to involve p53-mediated transcriptional regulation of members of the bcl-2 gene family (Halder *et al.* 1994, Miyashita *et al.* 1994, Selvakumaran *et al.* 1994, Zhan *et al.* 1994, Miyashita and Reed 1995, Kitada *et al.* 1996, Findley *et al.* 1997). On the other hand it has been reported that in

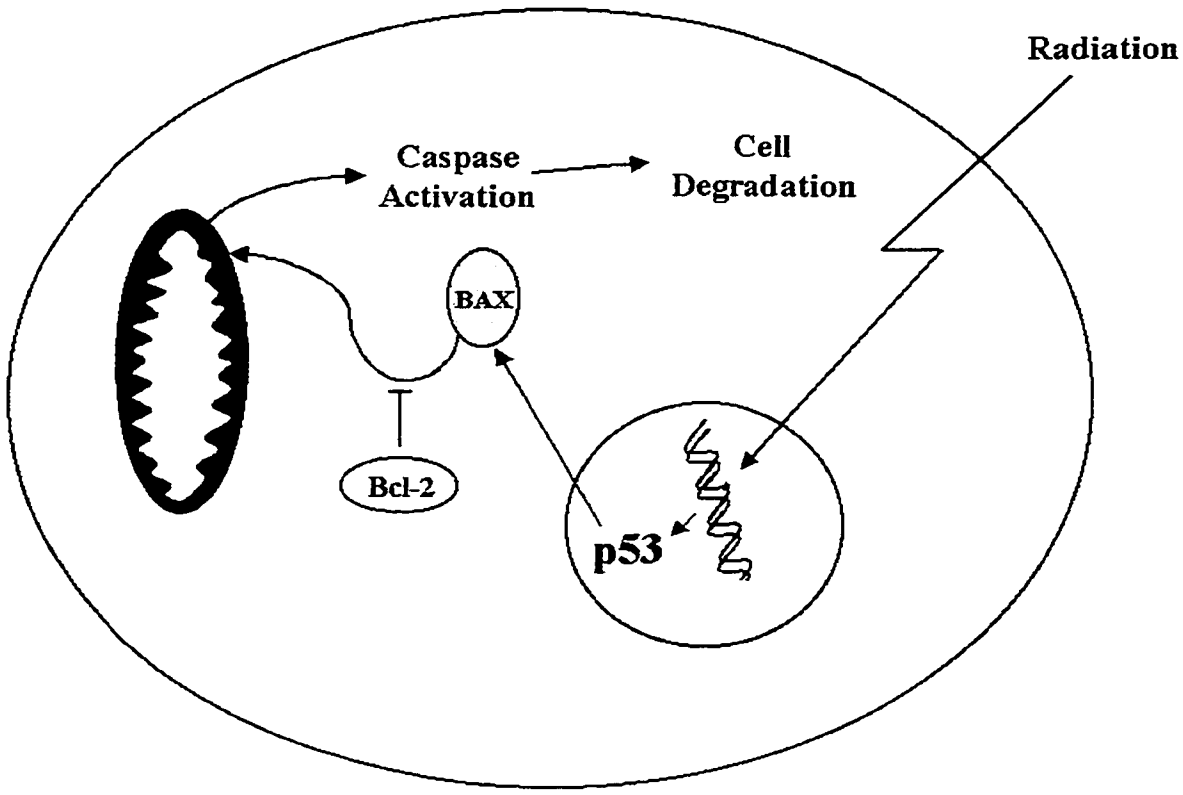

Figure 4. Genetic Regulation of Radiation Induced Apoptosis.

other circumstances nontranscriptional mechanisms may be involved (Caelles *et al.* 1994, Haupt *et al.* 1995, Bissonnette *et al.* 1997).

The involvement of p53 in radiation-induced apoptosis was first demonstrated in studies using thymocytes from mice in which the p53 gene had been homozygously inactivated by gene targeting. Whereas ionizing radiation could induce high levels of apoptosis in thymocytes from wild type mice,

thymocytes from mice deficient in p53 were found to be resistant to even high doses of radiation (Clarke et al. 1993, Lowe *et al.* 1993b). It was also shown in these studies that similar to ionizing radiation, other DNA damaging agents triggered apoptosis through a process requiring wild type p53 activity. This p53-dependent apoptotic pathway, however, appeared to be specific for DNA damaging agents since thymocytes from p53 null mice remained sensitive to the induction of apoptosis by nonDNA damaging agents such as calcium ionophores or dexamethasone. Since these initial studies, an essential role of p53 in radiation-induced apoptosis has also been reported in a number of other cell types including myeloid progenitor cells (Lotem and Sachs 1993), marrow pre-B cells (Strasser *et al.* 1994), quiescent peripheral B- and T- lymphocytes (Strasser *et al.* 1994), intestinal epithelial cells (Clarke *et al.* 1994, Merritt *et al.* 1994), and cerebellar granule neurons (Wood and Youle 1995, Enokido *et al.* 1996). A requirement for p53 in radiation-induced apoptosis has also been observed in cell types such as fibroblasts in which p53 activation normally results in cell cycle arrest (Kastan *et al.* 1992) and not apoptotic cell death. In a study by Lowe *et al.* (1993a) it was shown that while primary mouse embryo fibroblasts (MEFs) were resistant to DNA damage-induced apoptosis, MEFs transformed by adenovirus-derived E1A became sensitive to the induction of apoptosis by ionizing radiation as well as chemical DNA damaging agents such as 5-fluorouracil, etoposide, and adriamycin. Furthermore, this apoptotic cell death was found to be dependent on p53 since E1A expressing fibroblasts derived from p53 null mice remained resistant to drug or radiation-induced apoptosis. However, the necessity for p53

activation in radiation-induced apoptotic cell death is not universal. Cycling T lymphoma cells and mitogenically activated T lymphocytes from p53 null mice were shown to undergo apoptosis after irradiation or genotoxic drug treatment (Strasser *et al.* 1994, Tamura *et al.* 1995). Thus, p53-independent apoptotic pathways must also exist and other factors such as the prevailing growth conditions may influence which pathway is evoked.

Modification of Radiation-Induced Apoptosis

The susceptibility of cells to radiation-induced apoptosis can be influenced by a number of factors including cell type specific growth factors and cytokines. For example, radiation-induced apoptosis has been shown to be reduced in bovine endothelial cells in the presence of bFGF (Fuks *et al.* 1994), in bone marrow cells in the presence of interleukin-3 (Collins, M. *et al.* 1992), or in splenocytes after mitogen stimulation (Sellins and Cohen 1987). Cellular sensitivity to apoptosis has also been shown to be modified by inhibitors of certain signal transduction pathways. Phorbol esters such as TPA which are known to inhibit PKC activity have been shown to protect against radiation-induced apoptosis in a number of cell systems including endothelial cells (Haimovitz-Friedman *et al.* 1994), C3H 10T1/2 cells (Tomei *et al.* 1988), MOLT-4 leukemia cells (Akagi et al. 1993), LY-TH mouse lymphoma cells (Meyn *et al.* 1993), mouse thymocytes (Ojeda *et al.* 1992), and human lymphocytes (Tomei *et al.* 1990). Furthermore, phosphatase inhibitors such as okadaic acid or calyculin A have been shown to protect against radiation-induced apoptosis in

certain leukemia or lymphoma cell lines (Baxter and Lavin 1992, Song and Lavin 1993). Sensitivity to radiation-induced apoptosis has also been shown to be altered by enforced expression of certain proto-oncogenes. Overexpression of *c-myc* has been shown to promote radiation-induced apoptosis in transfected rat embryo cells (Chen *et al.* 1994) whereas transfection with *c-Ha-ras* resulted in increased radioresistance (McKenna *et al.* 1991, Cheong *et al.* 1993, Chen *et al.* 1994).

Radiation-Induced Apoptosis and Cancer

The involvement of known oncogenes and tumour suppressor genes in the regulation of apoptotic cell death suggests a link between the processes of apoptosis and carcinogenesis. For example, constitutive expression of the bcl-2 proto-oncogene, as a result of a translocation which places the bcl-2 gene in the vicinity of the immunoglobulin splice site, has been implicated as a major contributing factor in certain forms of B-cell lymphoma (Tsujimoto *et al.* 1984). Unlike most oncogenes, however, overexpression of bcl-2 does not act by promoting cell proliferation but rather by preventing apoptotic cell death (Vaux *et al.* 1988). Furthermore, as previously mentioned, overexpression of bcl-2 has been shown to protect against apoptotic cell death induced by radiation and other genotoxic agents (Strasser *et al.* 1994). Another gene common to apoptosis and cancer is the tumour suppressor gene p53. The p53 gene has been the most commonly identified mutated gene in human cancers and has been implicated in cancers of diverse origin (Hollstein *et al.* 1991, Levine *et al.*

1993). Mice in which the p53 gene has been homozygously inactivated by gene targeting are highly prone to developing cancer (Donehower *et al.* 1992, Jacks *et al.* 1994) and various cells derived from these animals have been shown to be resistant to the induction of apoptosis by genotoxic agents including ionizing radiation (Clarke *et al.* 1993, Lowe *et al.* 1993b, Lotem and Sachs 1993, Lee and Bernstein 1993, Merritt et al. 1994). Thus, mutations within the p53 gene may not only increase the likelihood of neoplasia, but may also make the resulting tumours more resistant to radiation- or chemotherapeutic treatments.

It is generally thought that apoptosis is triggered in cell populations exposed to radiation and other genotoxic agents as a means of eliminating cells containing residual genetic damage (Kemp et al. 1994, Norimura *et al.* 1996, Kondo 1998). This is proposed to serve an antineoplastic function by preventing the expansion of potentially malignant cells. Support for this hypothesis has come from studies comparing the radiation response of TK6 human lymphoblastoid cells and the derived daughter cell lines WI-L2-NS and WTK1, which contain mutations within the p53 gene. TK6 cells were found to be more sensitive to radiation-induced apoptotic cell killing and interestingly, showed significantly fewer chromosome aberrations and mutations after irradiation as compared with the p53-mutant daughter cell lines (Schwartz *et al.* 1995, Xia *et al.* 1995, Zhen *et al.* 1995).

Adaptive Response to Ionizing Radiation

The ability of cells to induce cellular repair processes, following exposure to mutagens at low concentrations, was originally demonstrated by Samson and Cairns (1977). They reported that exposure of *Escherichia coli* cells to a low concentration of MNNG could reduce the mutagenic and lethal effects induced by a subsequent (high concentration) MNNG exposure. This effect was not observed when cells were incubated in the presence of the protein synthesis inhibitor chloramphenicol, leading the authors to conclude that the low dose treatment induced an error-free DNA repair process. Consistent with this hypothesis, it was shown in later studies that this response was accompanied by an increased expression of the DNA repair enzymes O^6-methylguanine-DNA-methyl transferase and 3-methyladenine-DNA-glycosylase II (Lindahl *et al.* 1988). Pre-exposure to low concentrations of alkylating agents has also been shown to reduce subsequent drug induced cytogenetic damage in plant cells (Heindorff *et al.* 1987) and mammalian cells (Samson and Schwartz 1980). This phenomenon in which exposure of cells to a low dose of mutagen decreases the severity of the deleterious effects induced by a subsequent high dose of the mutagen has been termed "adaptive response" (*reviewed in* Frosina and Abbondandolo 1985).

In addition to being induced by chemical mutagens, an adaptive response has also been reported to occur in response to low doses of ionizing radiation (Mitchel and Morrison 1984). The induction of an adaptive response to ionizing radiation has since been demonstrated in human cells (Olivieri et al. 1984,

Shadley and Wolff 1987). In these studies it was shown that pretreatment of human lymphocytes *in vitro* to a low concentration of tritiated (radioactive) thymidine or a low dose of X-rays could reduce the frequency of chromosomal aberrations induced by a subsequent X-ray exposure (Olivieri *et al.* 1984). It was proposed that the low dose radiation exposure induced a cellular repair process which reduced the extent of chromosomal damage caused by the subsequent radiation exposure (Olivieri *et al.* 1984). This hypothesis was supported by the finding that the adaptive response to ionizing radiation could be inhibited by cycloheximide (Youngblom *et al.* 1989), suggesting a role for *de* novo protein synthesis, and by 3-amino-benzamide (Wiencke *et al.* 1986, Ikushima 1987, Shadley and Wolff 1987, Seong *et al.* 1995), an inhibitor of poly (ADP-ribose) polymerase which is thought to be involved in the repair of DNA strand breaks (Oleinick and Evans 1985). This DNA repair process was considered to be error-free since radiation adapted cells also showed a reduced frequency of mutations (Sanderson and Morley 1986, Kelsey *et al.* 1991, Zhou *et al.* 1993, Rigaud *et al.* 1993) and transformation to malignancy (Azzam *et al.* 1994).

Characteristics of the Adapting Radiation Exposure

Characterization of the adapting conditions in lymphocytes showed that induction of the adaptive response is dependent both on the total dose of the pretreatment and the dose rate at which it is given (Shadley and Wiencke 1989). The response was found to be induced by a narrow range of adapting doses

above or below which the response did not occur. Furthermore, it appeared that higher adapting doses must be given at a lower dose rate in order to be effective at triggering the adaptive process. The authors interpreted this to imply that a certain amount of damage needed to be induced within a specific period of time in order to elicit the adaptive response. Other studies have demonstrated that, in lymphocytes, a minimum time of 4-6 hours is required between the adapting and challenge radiation exposures in order to achieve full induction of the adaptive process (Shadley *et al.* 1987, Youngblom *et al.* 1989, Wang *et al.* 1991). Furthermore, it has been shown that the adaptive response, once induced, persists for approximately 3 cell cycles (Shadley *et al.* 1987, Cai and Liu 1990), although others have reported shorter durations (Wang *et al.* 1991). Other investigators have indicated an inverse correlation between the dose of the pretreatment and the duration of the adapting effect (Farooqi and Kesavan 1993).

The Effect of Cell Cycle on the Adaptive Response

The adaptive response to ionizing radiation also appears to be dependent on the stage of the cell cycle in which the adapting exposure is given. It has been demonstrated that the adaptive response could be induced in lymphocytes when the adapting dose was delivered to cells during G_1 (Wang *et al.* 1991, Shadley and Dai 1992) or S-phase (Shadley *et al.* 1987, Cai and Liu 1990) of the cell cycle. However, conflicting reports have been published with respect to the induction of the adaptive response when the adapting exposure is given in the

G_0 stage of the cell cycle. Early investigations by Shadley *et al.* (1987) failed to demonstrate an adaptive response when cells were pre-exposed to a low dose in G_0. This observation was confirmed in subsequent studies by Moquet *et al.* (1989) and Wang *et al.* (1991). However, other groups have indicated that the adaptive response to ionizing radiation could be induced when the adapting exposure was delivered in G_0 (Sanderson and Morley 1986, Cai and Liu 1990, Khandogina *et al.* 1991). Interestingly, an adaptive response has been observed in lymphocytes from individuals occupationally exposed to low doses of ionizing radiation (Tuschl *et al.* 1980; 1983, Liu *et al.* 1987, Barquinero et al. 1996, Gourabi and Mozdarani 1998) when the majority of lymphocytes are in the resting phase, ie. G_0.

Inter- and Intraindividual Variability of the Adaptive Response

Variability in the adaptive response to ionizing radiation has been reported for lymphocytes from different individuals (Bosi and Olivieri 1989, Sankaranarayanan *et al.* 1989). Initially, it was assumed that the inability of certain donors to exhibit an adaptive response was genetically determined and that these donors would be constitutively deficient for the induction of the adaptive response to ionizing radiation. Consistent with this hypothesis lymphocytes from certain individuals failed to demonstrate an adaptive response in repeat experiments (Bauchinger *et al.* 1989). However, other studies indicated that lymphocytes from certain individuals who did not previously exhibit an adaptive response did demonstrate an adaptive response on other occasions

(Olivieri and Bosi 1990). Thus, it was proposed that the ability to induce an adaptive response could be at least partially determined by the physiological state of the cell at the time of the low dose exposure (Olivieri and Bosi 1990). This was supported by the finding that addition of growth factors or hormones to the culture medium could modify the cellular response to the low dose radiation exposure in such a way that certain nonresponders could then exhibit an adaptive response (Olivieri and Bosi 1990, Shadley 1991). Furthermore, it was shown that the ability of lymphocytes to express an adaptive response could be influenced by altering the pH of the culture media (Bosi *et al.* 1991). In contrast, Hain *et al.* (1992) found no adaptive response in lymphocytes of several individuals under a variety of culture conditions. Therefore, the variability of the adaptive response observed in lymphocytes from different donors is likely to involve both genetic as well as physiological components.

Different Cell Types Exhibiting an Adaptive Response to Ionizing Radiation

The majority of studies concerning the adaptive response to ionizing radiation in mammalian cells have involved mitogen stimulated lymphocytes and have used chromosomal aberrations (specifically chromatid deletions) as an endpoint. More recently it has been demonstrated that the adaptive response to ionizing radiation also occurs in cultured human fibroblasts (Azzam *et al.* 1992), mouse SR-1 cells (Zhou *et al.* 1993), mouse bone marrow cells (Fomenko *et al.* 1991, Farooqi and Kesevan 1993), mouse spermatocytes (Cai and Liu 1990), cultured Chinese hamster cells (Ikushima 1989), C3H 10T1/2 cells (Azzam *et al.*

1994), as well as in different hepatoma and lymphoblastoid cell lines (Seong *et al.* 1995). Furthermore, in addition to aberrations, an adaptive response has also been demonstrated using other chromosomal damage endpoints such as sister chromatid exchanges (Moquet *et al.* 1989, Ikushima 1989), micronuclei (Ikushima 1987, Azzam *et al.* 1992; 1994), and gene mutations (Sanderson and Morley 1986, Kelsey *et al.* 1991, Zhou *et al.* 1993, Rigaud *et al.* 1993). Still other studies have used cell survival as an endpoint, however the results have been somewhat conflicting. Azzam *et al.* (1992) and Meyers *et al.* (1992) each observed a survival adaptive response whereas others have found no such survival advantage (Sanderson and Morley 1986, Rigaud *et al.* 1993) or have reported inconclusive results (Shadley and Dai, 1992).

Protein Induction in the Adaptive Response

It was shown that the adaptive response was blocked when cells were incubated in the presence of cycloheximide suggesting that active protein synthesis is required in the adaptive process (Youngblom *et al.* 1989). However, this inhibitory effect occurred only when cycloheximide was present during a critical time period of 4-6 hours after the low dose radiation exposure. The authors concluded that some proteins critical to the adaptive process are synthesized during this 4-6 hour time interval. Interestingly, this period coincides with the minimal time required between the adapting and challenge radiation exposures for the induction of the adaptive response to occur (Shadley *et al.* 1987, Wang *et al.* 1991). Studies by Wolff *et al.* (1989) and Ikushima (1992)

demonstrated that specific proteins were reproducibly induced by low doses of ionizing radiation (0.01Gy). The function of these proteins, however, were not characterized in these studies. Several other investigators have demonstrated the induction of specific mRNAs in response to low doses of ionizing radiation (Woloschak and Chang-Liu 1990, Meyers *et al.* 1992, Amundson et al. 1999). Some of these transcripts are known to code for proteins involved in cell cycle regulation such as p53. Consistent with this, recent studies have indicated that increases in cell cycle delays may contribute to the adaptive process (Meyers *et al.* 1995, Mitchel 1995, Amundson et al. 1999) presumably by allowing more time for damage to be repaired prior to fixation of the damage during mitosis.

Induction of the Adaptive Response by Radiomimetic Chemicals

In certain studies radiomimetic chemicals have been substituted for the adapting radiation exposure. These studies have demonstrated that the frequency of chromosomal aberrations induced by a challenge exposure of X-rays is significantly reduced in cells that had been pre-exposed to low concentrations of the free radical generating agents bleomycin (Vijayalaxmi et al. 1989, Wolff *et al.* 1989) or hydrogen peroxide (Cortes *et al.* 1990, Dominguez *et al.* 1993). Similar to the situation with adapting exposures of radiation, these chemicals were found to be effective at inducing the adaptive response only when used over a specific concentration range. Furthermore, cells pre-exposed to adapting concentrations of these radiomimetic chemicals exhibited a similar

reduction in subsequent radiation-induced chromosomal aberrations as cells pre-exposed to low doses of ionizing radiation.

In order to better characterize the repair systems induced in the adaptive response to ionizing radiation, certain investigators have tested the relative ability of radiation adapted cells to repair DNA lesions produced by different classes of DNA damaging agents. In this respect, Wolff *et al.* (1988) demonstrated that pre-exposure to a low dose of radiation could reduce the frequency of aberrations induced by a subsequent challenge exposure of bleomycin or mitomycin C. On the other hand, they showed that an adaptive response did not occur when cells were challenged with the alkylating agent methyl methane sulphonate (MMS). In another study the level of sister chromatid exchanges induced by a challenge exposure of 1,3-bis(2-chloroethyl)-1-nitrosurea (BCNU), cisplatin, or etoposide was shown to be reduced in lymphocytes which had been pre-exposed to a low dose of X-rays (Shadley and Dai 1993). These agents are known to produce chromosomal damage by different mechanisms suggesting that the repair process induced by the adapting dose of radiation is involved in the repair of a variety of DNA lesions. However, the lack of an adaptive response observed in cells challenged with MMS suggests that this repair process is not involved in the repair of chromosomal damage caused by alkylating agents.

The Effect of the Adaptive Response on Mutagenesis

Several research groups have investigated the fidelity of the induced repair response by examining radiation-induced mutagenesis in adapted cells. In

this respect, the frequency of radiation-induced mutations at the hypoxanthine phospho-ribosyltransferase (*hprt*) locus was found to be significantly reduced in lymphocytes which had been pre-exposed to a low dose of X-rays (Kelsey *et al.* 1991) or tritiated thymidine (Sanderson and Morley 1986). Similarly, it was shown that exposure of mouse SR-1 cells to a low dose of γ-rays rendered these cells less susceptible to the induction of mutagenesis by a subsequent exposure to either radiation or bleomycin (Zhou *et al.* 1993). Furthermore, in a study using lymphoblastoid cells, the authors demonstrated that in addition to decreasing the mutation frequency, pre-exposure to low dose ionizing radiation also resulted in a change in the type of mutational lesions (Rigaud and Moustacchi 1994). They reported that in unadapted cells *hprt* mutations are primarily gene deletions. Conversely, in adapted cells they were predominately gene rearrangements. In an extension of these studies, Azzam *et al.* (1994) demonstrated that pre-exposure of C3H 10T1/2 mouse embryo cells to a low dose of radiation could reduce the frequency of radiation-induced neoplastic transformation and of spontaneous neoplastic transformation in the absence of further radiation exposure (Azzam et al. 1996). In all of these studies it was concluded that the adaptive response to radiation involved the induction of an error-free DNA repair system. However, in some of these studies cell survival was also examined and was shown to be unaffected by pre-exposure to the adapting dose of radiation (Sanderson and Morley 1986, Rigaud *et al.* 1993, Azzam *et al.* 1994). Thus, it was proposed that mutation and lethality result from different types of DNA

lesions, and that low dose radiation induces a system which is involved in the repair of premutational lesions rather than potentially lethal lesions.

OBJECTIVES OF THE STUDY

Apoptosis is a genetically programmed cell death process which can be activated in cells exposed to a variety of toxic agents including ionizing radiation. The induction of apoptosis in response to radiation exposure is thought to function in addition to DNA repair processes by eliminating cells in which DNA damage has been misrepaired or incompletely repaired. Thus, according to this model the processes of DNA repair and apoptosis can cooperate to reduce the risk of carcinogenesis associated with radiation exposure by eliminating genetic damage in the exposed cell population (Figure 5). Implicit in this model is the assumption that radiation-induced apoptosis is triggered in response to DNA damage, a notion which remains controversial. Thus, the objective of **Chapter 1** was to test the hypothesis that *radiation-induced apoptosis is triggered in response to DNA damage*.

According to the proposed cellular response to radiation-induced DNA damage model (Figure 5), an enhanced induction of either an error-free DNA repair pathway or the enhanced removal of genetically damaged cells through apoptosis could reduce the risk of radiation-induced carcinogenesis. In this respect, an adaptive response to ionizing radiation has been reported in which exposure of cells to a low dose of ionizing radiation can lead to the enhanced induction of DNA repair systems which decreases the extent of chromosomal

damage induced by a subsequent radiation exposure. Since apoptotic cell death is also known to be a modifiable cellular process, I tested the hypothesis in **Chapter 2** that *the cellular sensitivity to radiation-induced apoptosis could be modified in cells pre-exposed to a low dose of ionizing radiation.*

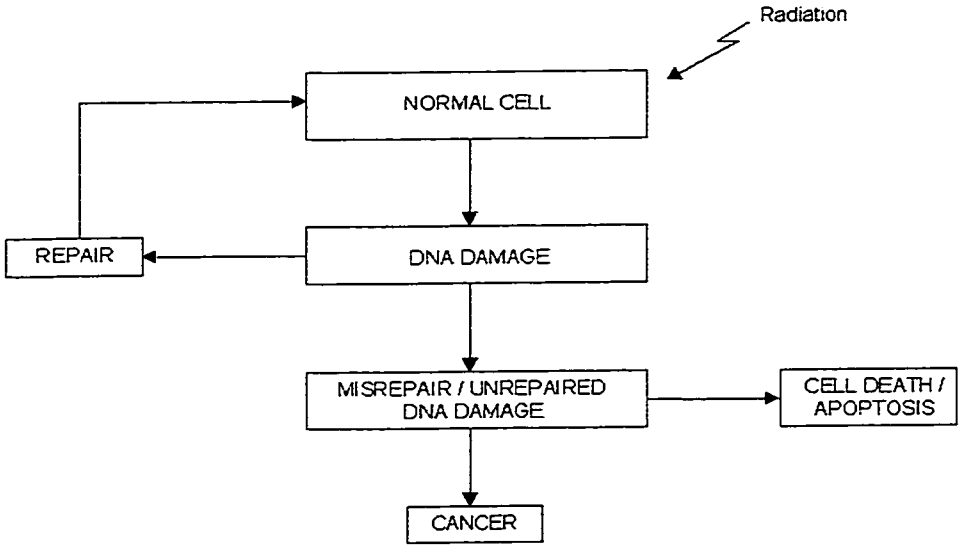

Figure 5. Cellular Response to Radiation-induced DNA Damage.

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CHAPTER 1

RADIATION-INDUCED APOPTOSIS IN HUMAN LYMPHOCYTES IS TRIGGERED BY DNA DAMAGE

INTRODUCTION

Ionizing radiation is known to interact with cells producing a variety of molecular lesions including lipid peroxidation of membranes (Koteles 1986) and oxidation of proteins (Stadtman 1993) as well as oxidative damage to DNA resulting in base modifications and strand breaks (Ward 1988). However, most of this damage is thought to be of little consequence in regard to clonogenic cell killing in which the lethal lesions are considered to be unrepaired or misrepaired DNA double strand breaks (Joshi *et al.* 1982, Iliakis 1991).

In certain cell types, however, radiation is known to trigger a different mode of cell death known as apoptosis (*reviewed in* Szumiel 1994). In contrast to the passive nature of radiation-induced clonogenic cell death, apoptosis involves an active cellular response to radiation-induced damage. In response to this damage the cell initiates a well coordinated degradation process resulting in characteristic biochemical and morphological cellular features (*reviewed* in Wyllie 1992). The most prominent changes occur in the nucleus where activation of specific endonucleases and proteases mediate cleavage of DNA into distinct fragments and result in the subsequent collapse of chromatin structure (*reviewed in* Earnshaw 1995).

Unlike the established role of DNA damage in radiation-induced clonogenic cell killing, the molecular lesions involved in the triggering of apoptotic cell death remain unclear. The literature indicates conflicting views concerning the critical targets involved in radiation-induced apoptosis, with certain reports favoring a DNA damage induced process while others favor a cell membrane-triggered response.

A role for DNA damage in the induction of apoptosis is supported by studies showing that the induction of apoptotic cell death by various genotoxic agents including ionizing radiation is controlled in murine lymphocytes by the tumour supressor gene p53 (Clarke *et al.*1993, Lowe *et al.* 1993, Strasser *et al.* 1994). The expression of p53 has been shown to be specifically induced in response to DNA damage (Fritsche *et al.* 1993, Nelson and Kastan 1994) suggesting a correlation between DNA damage induced p53 expression and the triggering of apoptotic cell death in these cells. However, the induction of apoptosis by dexamethasone, ionomycin, or phorbol esters, which are not known to directly damage DNA, did not depend on p53 function (Clarke *et al.* 1993, Strasser *et al.* 1994).

Further evidence supporting DNA as the critical target in radiation-induced apoptosis comes from studies using (I-125) radiolabeled iododeoxyuridine (^{125}IURD). Iodine-125 is known to cause localized radiation damage and incorporation of ^{125}IURD into cellular DNA is known to be efficient at producing DNA double-strand break damage (Radford and Hodgson 1985). Murine lymphoid cell lines known to undergo radiation-induced apoptosis were found to

be similarly sensitive to DNA double-strand breaks produced by an external γ-radiation source or by incorporation of ^{125}IURD (Radford 1991, 1994). In another study using a murine T-cell hybridoma , it was shown that incorporation of bromodeoxyuridine into cellular DNA could enhance both radiation-induced DNA damage and subsequent apoptotic cell death to a similar extent (Warters 1992).

However, other reports suggest a possible role of radiation-induced membrane damage in the signalling of apoptosis. In one such report it was demonstrated that Trolox, a water soluble analogue of vitamin E, could inhibit radiation-induced apoptosis in murine thymocytes which the authors interpreted as implicating membrane oxidative damage in the triggering of apoptosis by ionizing radiation (Ramakrishnan *et al.* 1993). Another group has reported that radiation can trigger apoptosis in bovine aortic endothelial cells by direct stimulation of membrane associated sphingomyelinase (Haimovitz-Friedman *et al.* 1994, Fuks *et al.* 1995).

Human lymphocytes have previously been demonstrated to be sensitive to the induction of apoptosis by ionizing radiation (Payne *et al.* 1992, Delic *et al.* 1993, Cregan *et al.* 1994, Hertvelt *et al.* 1997, Vral *et al.* 1998) and therefore provide a suitable model for studies on the mechanisms of radiation-induced apoptotic cell death. In the present study the roles of cell membranes and DNA as targets in radiation-induced apoptosis were investigated using agents known to cause oxidative membrane damage or different types of DNA damage. The lipophilic oxidizing agents t-butyl hydroperoxide and cumene hydroperoxide were used to test the ability of oxidative membrane damage to trigger apoptotic cell

death. These agents have previously been shown to stimulate lipid peroxidation in cell membranes (van den Berg *et al.* 1988) and their cytotoxicity has been attributed to this effect (Masaki *et al.* 1989, Geiger *et al.* 1993). Similarly, the role of free radical mediated DNA damage in the triggering of apoptosis was investigated using the radiomimetic agent bleomycin which is known to intercalate within DNA and cause oxidative base damage and DNA strand breaks through the production of oxygen derived free radicals (Povirk 1991). The ability of DNA damage to stimulate apoptosis was also investigated using cisplatin, a DNA crosslinking agent (Dijt *et al.* 1988), and the topoisomerase II inhibitor m-amsacrine which is known produce DNA double strand breaks by inhibiting the ligation function of the enzyme (Smith 1990).

The critical site of free radical damage in radiation-induced apoptosis was investigated using the free radical scavengers dimethyl sulfoxide (DMSO) and α-tocopherol (vitamin E). The water soluble hydroxyl radical scavenger DMSO has been established as an effective radioprotector against clonogenic cell death *in vitro* (Roots and Okada 1975). Protection is thought to arise from the ability of DMSO to scavenge hydroxyl radicals produced during water radiolysis, thereby diminishing the indirect damage of critical biological molecules by ionizing radiation (Reuvers *et al.* 1973). Vitamin E, on the other hand, is a lipid soluble antioxidant which is proposed to scavenge lipid derived free radicals and to prevent lipid peroxidation reactions (Sies and Murphy 1991).

The role of radiation-induced DNA damage in the induction of apoptosis was also investigated using the DNA repair inhibitor cytosine arabinoside (Ara-

C). Ara-C is a nucleotide analogue which competes with endogenous nucleotides for insertion into DNA during repair resynthesis. The incorporation of Ara-C inhibits the excision repair process by terminating chain elongation and preventing DNA ligation (Collins 1987). Ara-C has previously been shown to inhibit the repair of UVC (Yew and Johnson 1979) or γ-radiation (Carson *et al.* 1986) induced DNA damage in human lymphocytes.

MATERIALS and METHODS

Cell Preparation

Peripheral blood from human donors was drawn by venepuncture into heparinized vacutainer tubes (Becton Dickinson). Mononuclear cells were isolated from whole blood by density gradient centrifugation on Histopaque-1077 (Sigma). Cells were washed twice with phosphate buffered saline (PBS: 0.137M NaCl, 2.68mM KCl, 6.4mM $Na_2HPO_4.7H_2O$, 1.47mM KH_2PO_4, pH 7.4), and in preparation for treatment were resuspended in RPMI 1640 media (Sigma) pH 7.4 at approximately $8x10^6$ cells/ml. In certain experiments, peripheral blood mononuclear cells were depleted of B-lymphocytes and monocytes by passage of cell suspensions through a T-cell enrichment column (R&D Systems). This isolation step produced a cell population consisting of greater than 90% T-lymphocytes as determined by the fraction of cells labelling positive for the T-cell specific CD3 antigen (anti-CD3 monoclonal antibody, Becton Dickinson).

Cell Culture

After treatment cells were cultured for apoptosis development by suspension in RPMI 1640 media (pH 7.4) supplemented with 10% fetal calf serum, 2mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (GIBCO) at approximately $4x10^5$ cells/ml. Cell suspensions were transferred to T25 flasks and incubated at 37°C in a humidified atmosphere of 95% air, 5% CO_2.

Irradiations

Cell suspensions were irradiated at a dose rate of approximately 1.0 Gy/min using a ^{60}Co γ-ray source (Gamma cell 200, AECL Research). Cells were irradiated at either 0°C or 37°C , as indicated, by immersing sample tubes in an insulated glass beaker containing either ice-water or water at 37°C.

Drug Treatments

Bleomycin sulphate, tert-butyl hydroperoxide, cumene hydroperoxide, dexamethasone, m-amsacrine (m-AMSA), cycloheximide, α-tocopherol acetate (vitamin E), and cytosine arabinoside (Ara-C) were all obtained from Sigma and cisplatin was purchased from Bristol Myers Squibb. Vitamin E was prepared as a 50mM stock solution in ethanol and m-AMSA as a 2.5mM stock in 50% DMSO. Stock solutions of all other agents were prepared in highly purified water (milli-Q Systems). Stock solutions of drugs were diluted in RPMI 1640 media prior to each experiment.

Aliquots of cell suspensions (approximately $4x10^6$ cells) were transferred to individual centrifuge tubes and samples were incubated at either 0°C or 37°C for 15 minutes prior to drug treatment. Drugs were then added at the indicated concentrations and samples were incubated for 30 minutes at 0°C in an ice-water bath or at 37°C in a temperature controlled circulating water bath. Control cells were incubated in drug-free media. At the end of the drug exposures, cells were centrifuged and washed twice in ice-cold media to remove unincorporated drug and then assayed either immediately for DNA damage or incubated in complete media at 37°C for the indicated times and assayed for apoptosis. In DNA repair experiments cells were incubated at 37°C in serum-free media for the allotted repair period.

Fluorimetric Analysis of DNA Unwinding (FADU) Assay

DNA strand break damage was measured using the Fluorimetric Analysis of DNA Unwinding (FADU) assay (Birnboim and Jevcak 1981). This method utilizes the phenomenon that the rate of unwinding of double-stranded DNA under alkaline conditions is porportional to the frequency of unwinding points (ie. single or double strand breaks). DNA strand breaks induced by drug or radiation treatments increase the rate of DNA unwinding and, consequently, decreases the amount of remaining double-stranded DNA. This change in unwinding rate is then reflected in a decrease in fluorescence of ethidium bromide added to the DNA extract.

Approximately $4x10^6$ cells were resuspended in 3ml of ice-cold inositol (0.25M meso-inositol, 10mM sodium phosphate, 1mM $MgCl_2$, pH 7.2) and aliquots of cell suspensions were transferred to three sets of four 16x100mm glass test tubes held in ice-water. The three sample sets were designated as **T**, **P** , and **B** to indicate total, partial, and background fluorescence respectively. Cell lysates were prepared by incubating all samples for 10 minutes at 0°C in lysis solution (9M urea, 10mM NH_4OH, 2.5mM CDTA, and 0.1% SDS). **P** samples were then subjected to an unwinding solution (50% lysis solution in 0.2N NaOH, pH 12.8) for 30 minutes at 0°C followed by a 60 minute incubation at 15°C. The unwinding of **P** samples was stopped by the addition of a neutralizing solution (1M glucose, 14mM mercaptoethanol) and sample tubes were sonicated (Branson sonifier) for 15 seconds. **B** samples were treated with unwinding solution, sonicated, and incubated for 30 minutes at room temperature to allow complete unwinding of DNA prior to the addition of the neutralizing solution. DNA in **T** samples is prevented from unwinding by simultaneous addition of the neutralizing and unwinding solutions prior to sonication. Ethidium bromide (4μg/ml) was then added to all tubes and the fluorescence intensity was measured using a spectrofluorimeter (excitation 520nm, emission 590nm). The extent of DNA unwinding for each treatment (D) is calculated from the fluorescent values of the **T**, **P**, and **B** samples (mean of four independent determinations for each) according to the formula D = (P-B) / (T-B). The relative amount of DNA damage in each sample was then expressed in Q_d units, where $Q_d = -100 \log (D_t/D_c)$ and D_t and D_c are the percentages of

double-stranded DNA (as determined fluorometrically) in treated and untreated cells respectively (McWilliams *et al.* 1983).

In Situ Terminal Deoxynucleotidyl Transferase (TdT) Assay

Apoptosis was detected in individual cells using a fluorescence in situ TdT assay (Apoptag, Oncor Inc.). During apoptosis cellular DNA is cleaved by endogenous nucleases producing DNA fragments with free 3'-OH ends. In this technique, digoxigenin labeled nucleotides are added to these 3'-OH sites by the enzyme *terminal deoxynucleotidyl transferase* (TdT). Cells undergoing apoptotic DNA fragmentation are then labelled using fluorescein-conjugated antidigoxigenin antibodies and scored by fluorescence microscopy.

Aliquots of cell suspensions (~$4x10^5$ cells) were washed twice in PBS and fixed for 10 minutes in 4% formalin in PBS (pH 7.4). Cells were deposited onto microscope slides using a Cytospin centrifuge (Shandon Inc., Pittsburgh, PA), air dried for 1 hour, and then stored in ethanol at -20°C. Cells were rehydrated in three changes (for 5 minutes each) of PBS prior to running the TdT assay.

Cells were permeabilized by incubating for 20 minutes in 0.2% Triton X-100 in PBS (pH 7.4). Slides were rinsed three times in PBS (4 minutes each) and then incubated for 10 minutes with Equilibration Buffer (Apoptag kit reagent). Excess reagent was removed and cells were covered with 30μl of TdT enzyme (Apoptag kit reagent) and incubated at 37°C for 60 minutes in a humid chamber. The enzyme was then inactivated by incubating for 30 minutes in Stop/wash Buffer (Apoptag kit reagent) followed by rinsing in 3 changes of PBS. Cells were

then incubated for 30 minutes with 26μl of antidigoxigenin- fluorescein (Apoptag kit reagent). Slides were washed in 3 changes of PBS and then counterstained for 2 minutes with 1μg/ml Hoechst 33258 in PBS. Slides were dried and mounted under glass coverslips with antifade mounting media (Oncor, Inc.) and stored at 4°C in the dark. Apoptotic cells labelled with fluorescein were counted on coded slides using a Zeiss Axiophot fluorescence microscope equipped with a fluorescein filter (520nm) and compared with the total number of cells counted that exhibited Hoechst fluorescence (395nm). A minimum of 1000 cells were scored per treatment.

Comet Assay

The comet assay or single cell gel electrophoresis technique provides a method for measuring DNA strand breaks in individual cells (Ostling and Johanson 1984). This technique has been used to measure drug or radiation-induced DNA damage (Singh *et al.* 1994) as well as subsequent DNA repair (Olive *et al.* 1990). It has also been used to detect apoptotic DNA fragmentation in individual cells (Olive *et al.* 1993, Fairnbairn *et al.* 1996). The technique involves the following basic steps: cells are embedded in agarose on a microscope slide, lysed, subjected to an electric field under alkaline conditions, and then stained with a fluorescent DNA binding dye and visualized by fluorescence microscopy. Under these electrophoretic conditions damaged DNA is able to migrate away from the nucleus into the "tail" region of the comet. The extent of migration is related to the relative size and number of DNA fragments.

Microgels were prepared as described elsewhere (Singh *et al.* 1994). Aliquots (200μl) of molten 1% low melting point (LMP) agarose (Gibco BRL) in PBS were layered onto fully frosted microscope slides (Fisher Scientific) and immediately covered with 24x50 mm coverslips. Slides were placed onto a cold steel tray at 4°C to allow agarose to gel and the coverslips removed after approximately 2 minutes. A second agarose layer was added to ensure minimal light diffusion from the frosted slides during microscopy. 20μl of LMP agarose was then mixed with an equal volume of cell suspension (approx. 10^4 cells) and a 20μl aliquot placed onto the middle of an agarose slide and covered with an 18x18 mm coverslip. Two additional samples were layered on either side of the center coverslip. After the agarose had solidified, the coverslips were removed and the samples were overlayed with an additional 200μl of agarose and allowed to set.

The slides were then immersed in lysis solution (1% sodium sarcosinate, 2.5M NaCl, 100mM Na_2-EDTA, 10mM Tris, pH 10) supplemented with 10% DMSO and 1% Triton X-100 for 45 minutes at 4°C. The slides were drained and immersed in alkali buffer (100mM NaOH, 0.33mM EDTA) for 30 minutes at 4°C and then subjected to electrophoresis in alkali buffer at 25V for 10 minutes. Slides were rinsed in excess neutralizing buffer (0.4M Tris, pH 7.4) and stained with the DNA specific dye YOYO-1 (Molecular Probes). A coverslip was applied and the slides were scored either immediately or after storage in a humidified atmosphere overnight at 4°C in the dark. Slides were visualized using a Zeiss Axiophot fluorescence microscope equipped with a fluorescein filter (520 nm).

Apoptotic cell frequencies were determined by blind scoring of comets as indicating undamaged or extensively damaged cells present on coded slides. A minimum of 500 cells were scored for each treated or control sample.

Pulsed Field Gel Electrophoresis

Pulsed Field gel electrophoresis (PFGE) was used in certain experiments to determine whether drug or radiation treatments induced a pattern of DNA fragmentation characteristic of apoptotic cell death (Cohen *et al.* 1994, Walker *et al.* 1994).

Detection of high-molecular-weight DNA fragmentation by PFGE was performed as previously described (Walker *et al.* 1993). Aliquots of cell suspensions were centrifuged and washed in nuclear buffer (NB) consisting of 60mM KCl, 15mM NaCl, 2mM EDTA, 1mM EGTA, 0.5mM spermidine, 0.15mM spermine, and 15mM Tris-HCl, pH 7.4. Cell pellets (1-2x10^6 cells) were then resuspended in NB and mixed with an equal volume of buffer containing 1.5% LMP agarose (BRL) and 0.2 mg/ml proteinase K at 37°C. The mixture was pipetted into the wells of a Biorad casting mould and allowed to set for 10 minutes at 4°C. Agarose plugs were then incubated for 3 hours at 37°C in TEEN buffer (10mMNaCl, 25mM EDTA, 5mM EGTA, and 10mM Tris-HCL, pH 9.5) supplemented with 0.5% SDS and 0.2 mg/ml proteinase K, and then washed twice for 30 minutes each at 4°C in TE buffer (1mM EDTA, 10mM Tris-HCl, pH 8.0). Agarose plugs and DNA size standards were then loaded on a 0.8% agarose gel in 0.5X TBE buffer (89mM Tris, 89mM boric acid, and 2.5mM

EDTA, pH 8.5). Electrophoresis was carried out using a FIGE unit (Bio-Rad) with 0.5X TBE buffer circulating at 14°C for 20 hours at 280V (forward)/90V (reverse) with a ramping time varying from 0.3 to 35s (forward)/ 0.5 to 60s (reverse). Gels were then stained for 30 min with ethidium bromide (1.5μg/ml) and destained for 1 hour. DNA size standards include yeast chromosome and Lambda ladder (New England BioLabs).

Measurement of Lipid Peroxidation using cisParinaric acid

Lipid peroxidation was measured by a previously described fluorimetric-based method (Kuypers *et al.* 1987) using the lipid soluble fluorescent probe cis-parinaric acid (Molecular Probes). Parinaric acid contains four conjugated double bonds, which render it naturally fluorescent. This alternating double bond structure is attacked during lipid peroxidation reactions resulting in the loss of fluorescence capacity. Similar with other fatty acids, parinaric acid readily incorporates into cellular membranes, and its loss of fluorescence over time has been used to monitor lipid peroxidation in cells (van den Berg *et al.* 1988, Hedley and Chow 1992, Makrigiorgios *et al.* 1997). In preliminary experiments, the fluorescence intensity of cells was found to be dependent on parinaric acid concentration, increasing linearly up to a concentration of approximately 10μM.The probe was used within this linear concentration range so as to directly correlate changes in fluorescence intensity with changes in probe oxidation levels.

A small volume of parinaric acid (final concentration of 10μM) was added to cells suspended in PBS at $2x10^6$ cells/ml and incubated for 30 minutes at 37°C. Cells were then centrifuged and resuspended in PBS ($2x10^6$ cells/ml) to remove unincorporated probe, and 3ml aliquots were transferred to individual glass tubes held at either 0°C or 37°C. Cells were then either irradiated or drug treated as previously described. Sample fluorescence was measured in acrylamide cuvettes (Fisher Scientific) using a spectrofluorimeter with excitation wavelength of 324nm (slit width 3nm) and emission wavelength of 418nm (slit width 10nm). Percent loss in fluorescence was calculated as I_0-I_{30} / I_0, where I_0 represents the sample fluorescence determined immediately prior to drug or radiation exposure and I_{30} the sample fluorescence measured 30 minutes after initiation of drug or radiation exposure.

Statistical Analysis

Data points represent the mean and standard deviation of 3 independent determinations unless indicated otherwise. Where applicable, means were compared using a Student's *t*-test for paired samples and the difference between two means was considered statistically significant if $p < 0.05$.

RESULTS

Time- and Dose-Dependency of Radiation-Induced Apoptosis in Human Lymphocytes

DNA strand breaks were measured in human lymphocytes incubated at 37°C for various lengths of time following exposure to 4Gy γ-radiation (Figure 1.1). The relative level of DNA strand breaks measured immediately after exposure to radiation at 0°C was found to be 47.4 ± 5.9 Q_d units. However, the number of DNA strand breaks decreased rapidly upon incubation at 37°C, presumably as a result of enzymatic repair of these initial radiation-induced DNA lesions. Residual DNA damage reached a minimum approximately 4 hours after radiation exposure when greater than 90% of the initial strand breaks had been repaired. At longer incubation periods, however, strand break damage began to rise again increasing from 3.8 ± 1.8 Q_d units at 4 hours to 20.1 ± 5.8 Q_d units at 24 hours. A similar result was observed when DNA damage was assessed in individual cells using the comet assay (Figure 1.2). Immediately following exposure to radiation at 0°C, DNA strand breaks could be observed in individual cells as an increase in DNA migrating from the nucleus into the "tail" region of the comet (compare Figures 1.2A and 1.2B). This comet "tail" fluorescence was

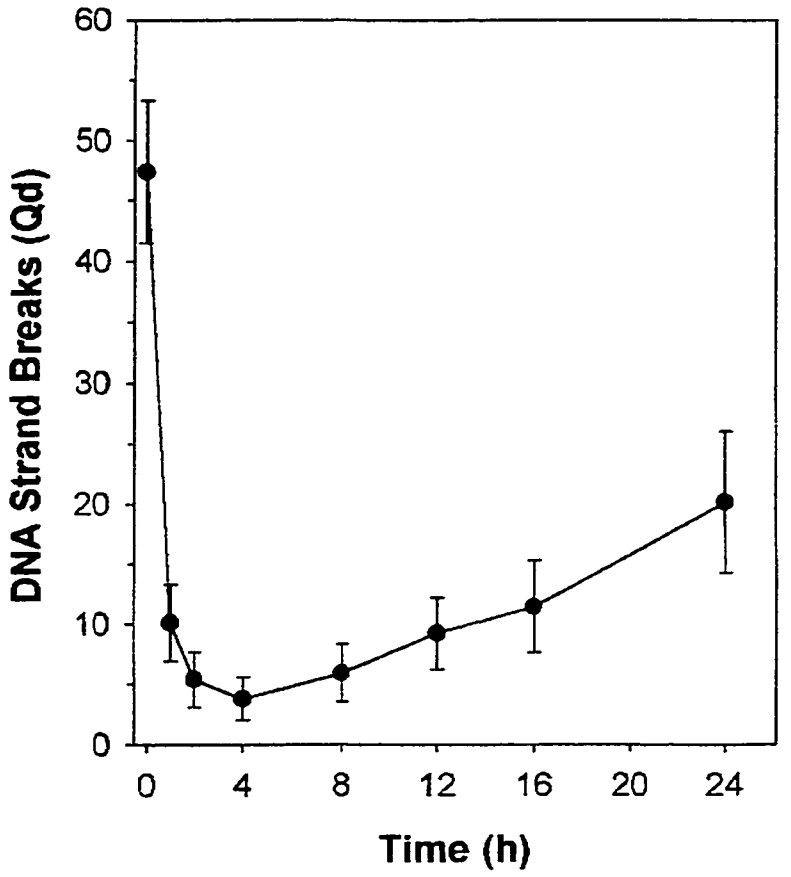

Figure 1.1. Radiation-Induced DNA Damage, Repair, and Reappearance of DNA Strand Breaks. DNA damage was measured using the FADU assay in lymphocytes exposed to 4 Gy γ-radiation and incubated at 37°C for the indicated times (n=5).

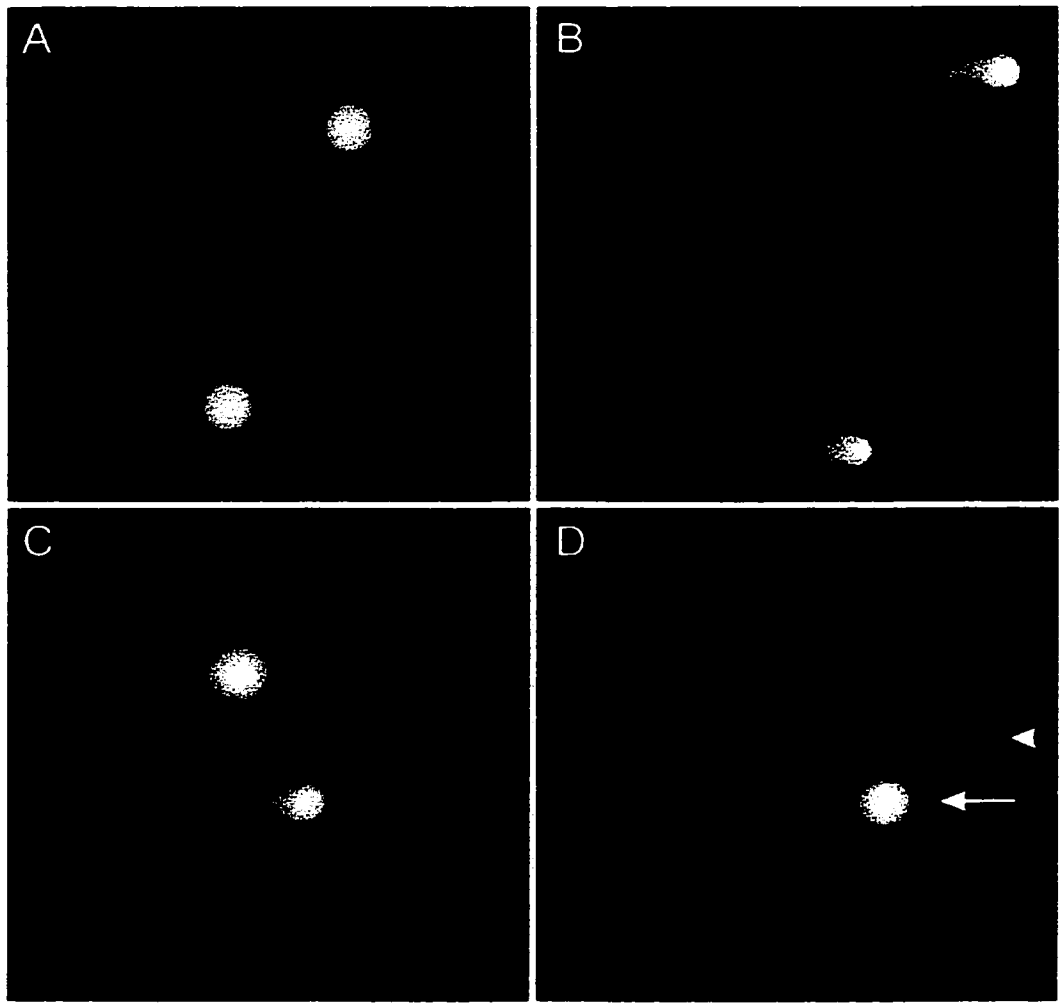

Figure 1.2. Radiation Induced DNA Damage, Repair, and Apoptotic DNA Fragmentation in Lymphocytes Detected by the Comet Assay. Photomicrographs of comets from unirradiated cells (A) or cells exposed to 4 Gy γ-radiation (at 0°C) and incubated at 37°C for 0, 4, or 24 hours (B-D). Panel A shows the appearance of typical control comets with very little DNA migrating away from the comet "head" (or nucleus). Panel B shows damaged DNA migrating into the comet "tail" immediately following exposure to radiation and Panel C shows the disappearance of this damage during subsequent incubation at 37°C. Panel D demonstrates the existence of two distinct cell populations at later times following exposure to radiation: control-like comets (arrow) and apoptotic comets (arrowhead) exhibiting extensive DNA fragmentation.

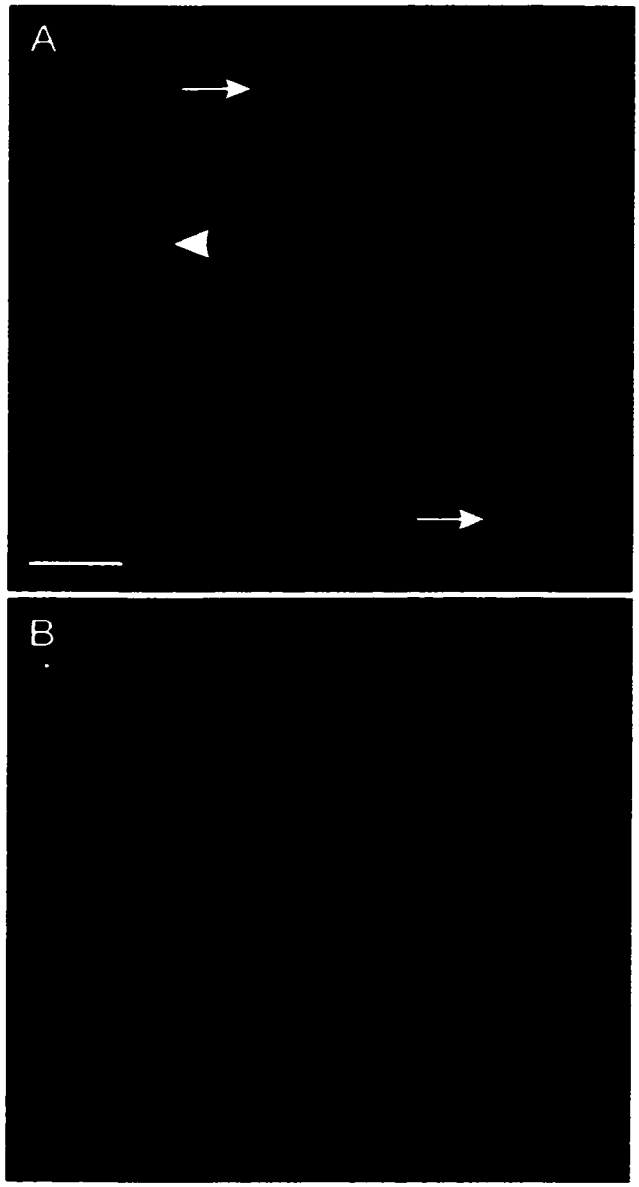

Figure 1.3. Lymphocytes Undergo DNA Fragmentation and Chromatin Condensation Characteristic of Apoptotic Cell Death Following Exposure to Ionizing Radiation. Photomicropgraphs of lymphocytes labelled in the TdT assay 24 hours after exposure to 4 Gy γ-radiation. Panel A. Hoechst 33258 counterstaining showing chromatin condensation typical of apoptotic nuclei (arrow) as compared with the diffuse staining pattern seen in nonapoptotic cells (arrowheads). Panel B. Same field showing FITC-labelling of DNA fragmentation specifically in cells exhibiting an apoptotic nuclear morphology. Magnification bar = 10μm.

markedly reduced when cells were incubated for 4 hours at 37°C following irradiation, indicating that DNA repair had occurred (Figure 1.2C). At later times, however, two distinct cell populations could be observed (Figure 1.2D): one population resembling control cells with little or no evident DNA damage (arrow), and another population exhibiting extensive DNA degradation (arrowhead) indicative of cells undergoing apoptotic DNA fragmentation (Olive et al. 1993, Fairbairn et al. 1996). Coincident with this reappearance of DNA damage, lymphocytes labeled in the TdT assay and examined by fluorescence microscopy revealed a population of cells exhibiting chromatin condensation and DNA fragmentation characteristic of apoptotic cell death (Figure 1.3).

The relationship between DNA strand break reappearance and apoptosis was further evaluated by studying the kinetics and dose dependency of the two processes (Figure 1.4). Figure 1.4A demonstrates the time dependency for the reappearance of DNA strand breaks (FADU assay) and apoptotic cells (TdT assay) following exposure to 4Gy γ-radiation. The frequency of apoptotic cells in untreated control samples reached a level of 9.8 $\pm$ 2.9% during the 72 hour incubation. In irradiated samples the percentage of apoptotic cells began to exceed control levels after approximately 12 hours and continued to increase with time, reaching a plateau between 48 and 72 hours post-treatment. This time-dependent increase in the fraction of apoptotic cells was paralleled by an increase in DNA strand break damage as measured by the FADU assay.

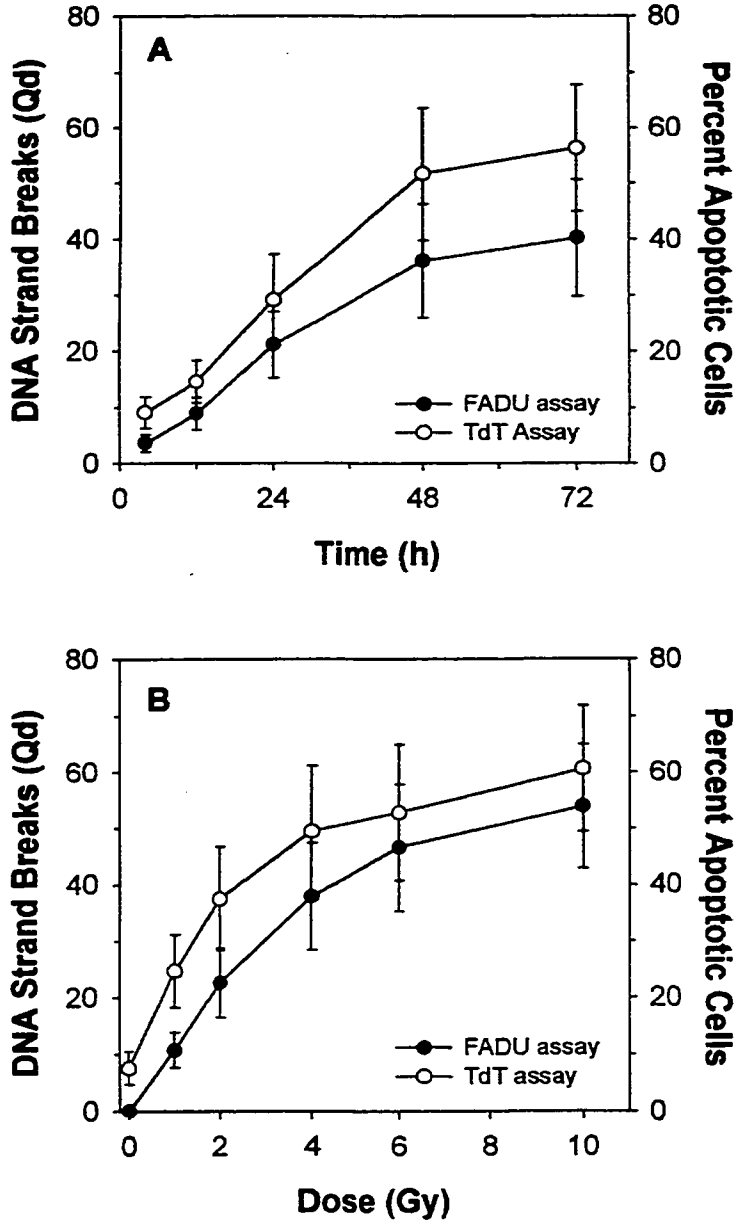

Figure 1.4. Comparison of FADU and TdT Assays for the Measurement of Radiation-Induced Apoptosis. Panel A. Lymphocytes were exposed to 4 Gy γ-radiation and assayed for DNA strand breaks and apoptotic cell frequency as a function of time after radiation exposure using the FADU and TdT assays, respectively (n=4). Panel B. Lymphocytes were exposed to increasing doses of γ-radiation and DNA strand breaks and apoptotic cell frequency was measured 48 hours later using the FADU and TdT assays (n=5).

In a second set of experiments lymphocytes were exposed to increasing doses of γ-radiation and the frequency of apoptotic cells and DNA strand break damage was measured 48 hours later by the TdT and FADU assays, respectively (Figure 1.4B). The percentage of apoptotic cells (TdT assay) was found to increase as a function of dose up to at least 10 Gy, although only a modest increase was observed at doses above 6 Gy. A similar dose responsive increase in DNA strand break damage was detected using the FADU assay. These results indicate that in addition to its ability to measure the initial radiation-induced DNA strand breaks (Birnboim and Jevcak, 1981), the FADU assay can be used to measure subsequent apoptotic DNA fragmentation in human lymphocytes (Cregan *et al.* 1994).

The Time- and Concentration-Dependent Induction of Apoptosis by Membrane or DNA Active Agents

The role of cell membranes or DNA as targets in radiation-induced apoptosis was investigated using agents known to cause oxidative membrane damage or different types of DNA damage. The ability of membrane oxidative damage to trigger apoptosis was tested by exposing lymphocytes to increasing concentrations of the lipophilic oxidizing agents t-butyl hydroperoxide or cumene hydroperoxide (Figure 1.5). Both of these agents were found to trigger apoptosis in a concentration dependent manner with the fraction of apoptotic cells exceeding 75% at the highest drug concentration tested (800μM). Similarly, the

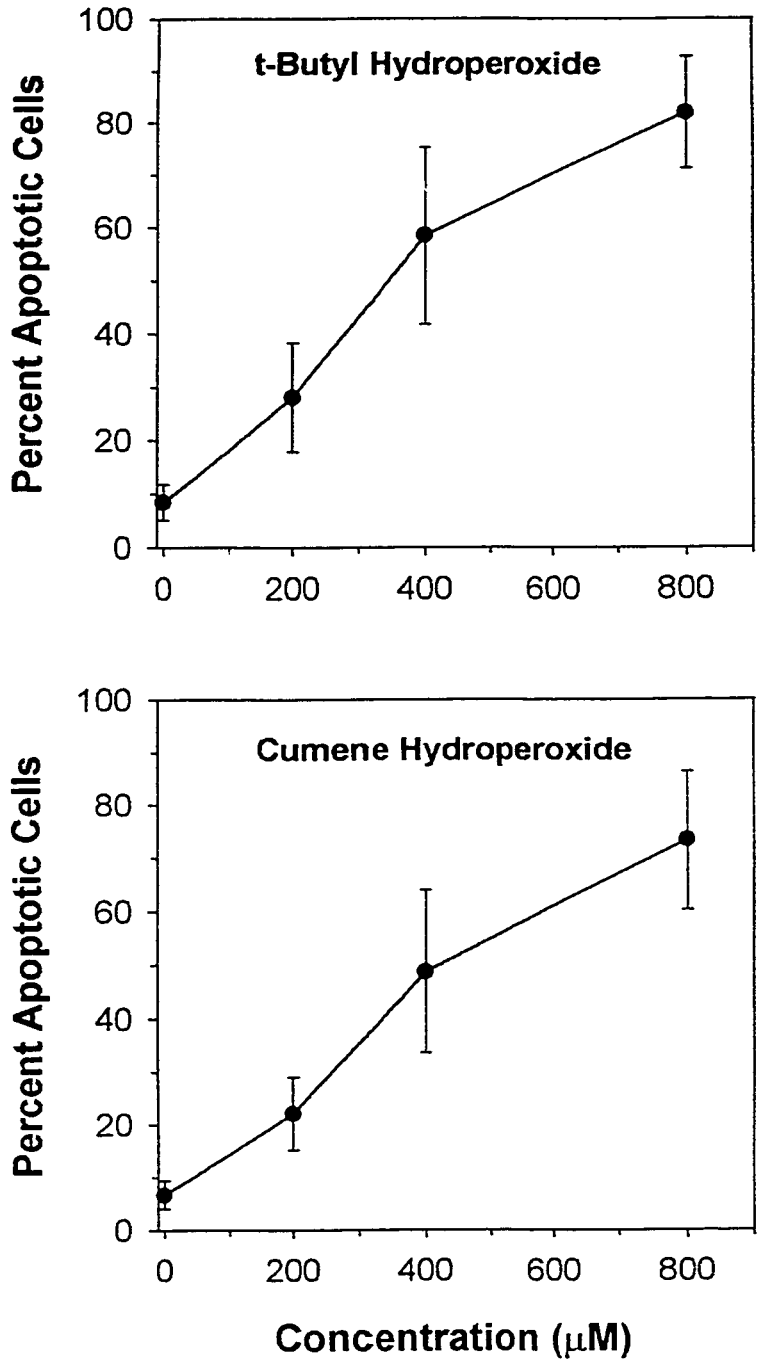

Figure 1.5. Dose Dependent Induction of Apoptosis in Lymphocytes Treated with Membrane Oxidizing Agents. Lymphocytes were treated with the indicated concentrations of t-butyl hydroperoxide or cumene hydroperoxide (30min, 37°C) and the fraction of apoptotic cells was measured 48 hours later using the TdT assay (n=3).

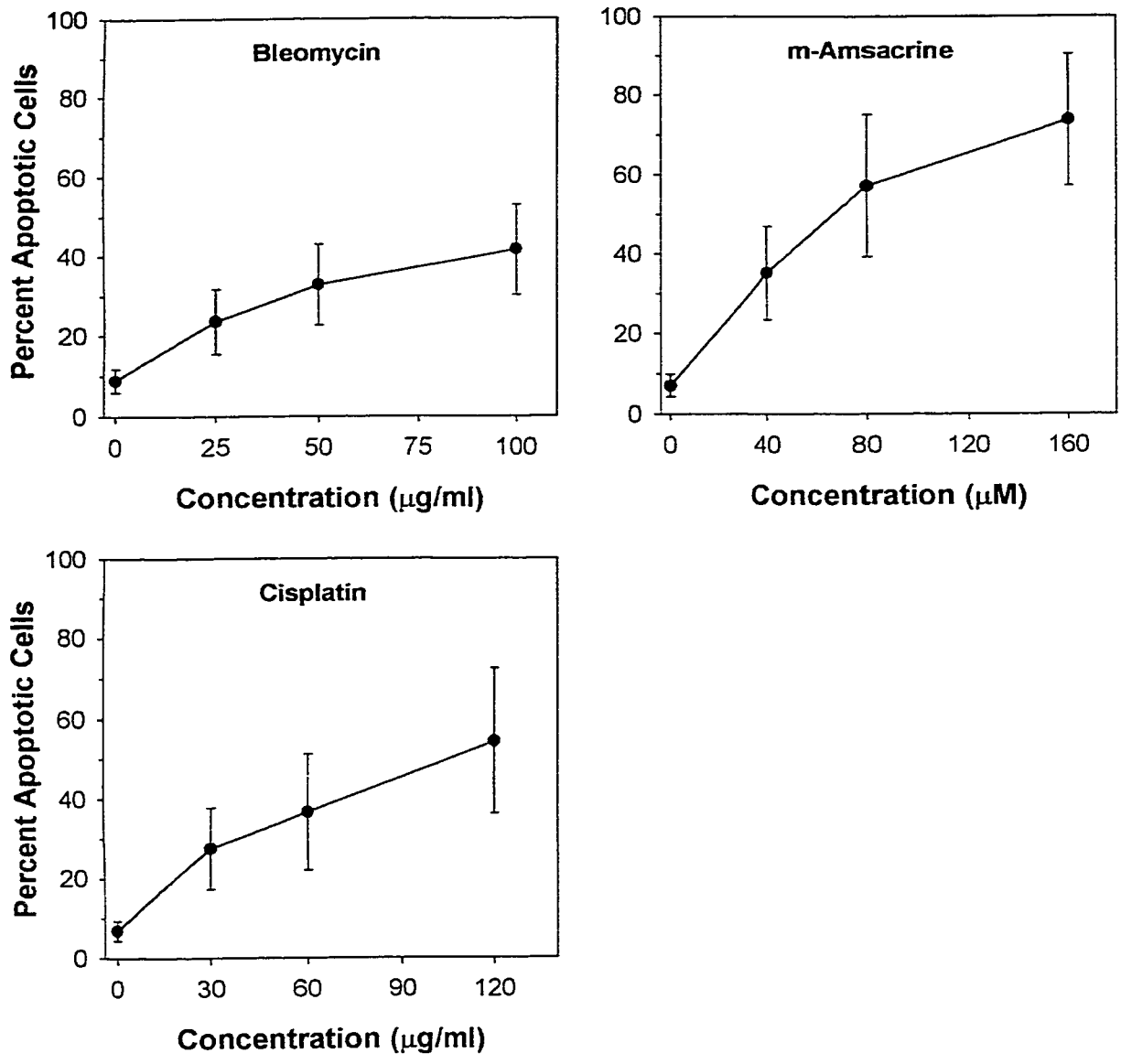

Figure 1.6. Dose Dependent Induction of Apoptosis in Lymphocytes Treated with DNA Damaging Agents. Lymphocytes were treated with the indicated concentrations of bleomycin, m-amsacrine, or cisplatin (30 min, 37°C) and the fraction of apoptotic cells was measured 48 hours later using the TdT assay (n=3).

ability of DNA damage to trigger apoptosis was examined by exposing lymphocytes to increasing concentrations of the genotoxic agents bleomycin, m-amsacrine, or cisplatin (Figure 1.6). These agents were also found to cause a dose responsive increase in the frequency of apoptotic cells. The topoisomerase II inhibitor m-amsacrine was found to be particularly effective and at the highest concentration tested (160μM) resulted in an apoptotic fraction of approximately 80%. The solvent carrier, DMSO, did not affect cell death even at the highest concentration used (0.25%).

When cells were exposed to drugs or radiation and apoptosis was measured as a function of time, the DNA damaging agents and membrane oxidizing agents were found to trigger apoptosis with distinctly different kinetics. The lipophilic oxidizing agents, t-butyl hydroperoxide and cumene hydroperoxide, triggered a rapid induction of the apoptotic process with maximal levels of apoptotic cells occurring within 2 hours of drug exposure (Figure 1.7). In contrast, the fraction of apoptotic cells did not begin to increase appreciably until approximately 12 hours after exposure to the DNA damaging agents bleomycin, m-amsacrine, or cisplatin and maximal levels were not achieved until 48-72 hours after drug exposure (Figure 1.8). Figure 1.8 also shows that the induction of apoptosis by ionizing radiation occurs with slow kinetics similar to that of the chemical DNA damaging agents

Endonucleases involved in apoptotic cell death are known to produce a characteristic pattern of high molecular weight DNA fragments which can be resolved by pulsed field gel electrophoresis (Oberhammer *et al.* 1993, Cohen *et*

al. 1994, Walker *et al.* 1994). Therefore, to confirm that cells were dying by apoptosis, DNA degradation was analyzed by PFGE at various times after drug or radiation exposure (Figure 1.9). Dexamethasone (Dex) was included as a positive control because of its known ability to trigger apoptotic DNA fragmentation without directly damaging DNA itself (Cohen and Duke 1984). All of the agents tested produced a similar pattern of DNA fragmentation consistent with apoptosis, including a prominent band at the 50kb position. Similar to the results shown in Figures 1.7 and 1.8, the time lapse between drug exposure and the appearance of apoptotic DNA fragmentation was found to differ for the various agents (Figure 1.9). The lipophilic oxidizing agent t-butyl hydroperoxide triggered a rapid induction of DNA fragmentation which was apparent within 1 hour of drug exposure. In contrast, DNA fragmentation following exposure to m-amsacrine or γ-radiation was not evident for at least 12 hours, and only became generally apparent at later times (48 hours).

To determine whether the kinetics of apoptosis might be dose-dependent, the kinetics of t-butyl hydroperoxide and γ-radiation-induced apoptosis were determined following exposure to increasing drug or radiation doses. Exposure to t-butyl hydroperoxide at a concentration of 200μM or 800μM resulted in approximately 30% and 80% apoptotic cells, respectively. Both drug concentrations, however, resulted in a rapid induction of the apoptotic process with maximal levels of apoptosis occurring within two hours of drug exposure (Figure 1.10A). Likewise, radiation-induced apoptosis occurred with slow kinetics following exposure to either 1Gy or 8Gy of γ-radiation despite a 3-fold

increase in the peak levels of apoptosis, 48 hours after exposure at these respective doses (Figure 1.10B). These results indicate that the different kinetics of the apoptotic response triggered by t-butyl hydroperoxide or γ-radiation is not likely the result of a dose related change in the kinetics of the apoptotic response.

Since human lymphocytes consist of various subpopulations of cells, it is possible that the different apoptotic kinetics induced by DNA damaging agents or lipophilic oxidizing agents could reflect responses of different cell populations. To test this possibility, the kinetics of t-butyl hydroperoxide or γ-radiation-induced apoptosis was determined in a cell population highly enriched in T-cells isolated from the population by antigen affinity chromatography. Similar to the results in the unseparated lymphocyte population (Figures 1.7 and 1.8), the apoptotic processes induced by t-butyl hydroperoxide or radiation in T-lymphocytes were found to occur with fast and slow kinetics respectively (Figure 1.11). This result suggests that the different apoptotic kinetics induced by these agents are not likely to be due to different subpopulation responses.

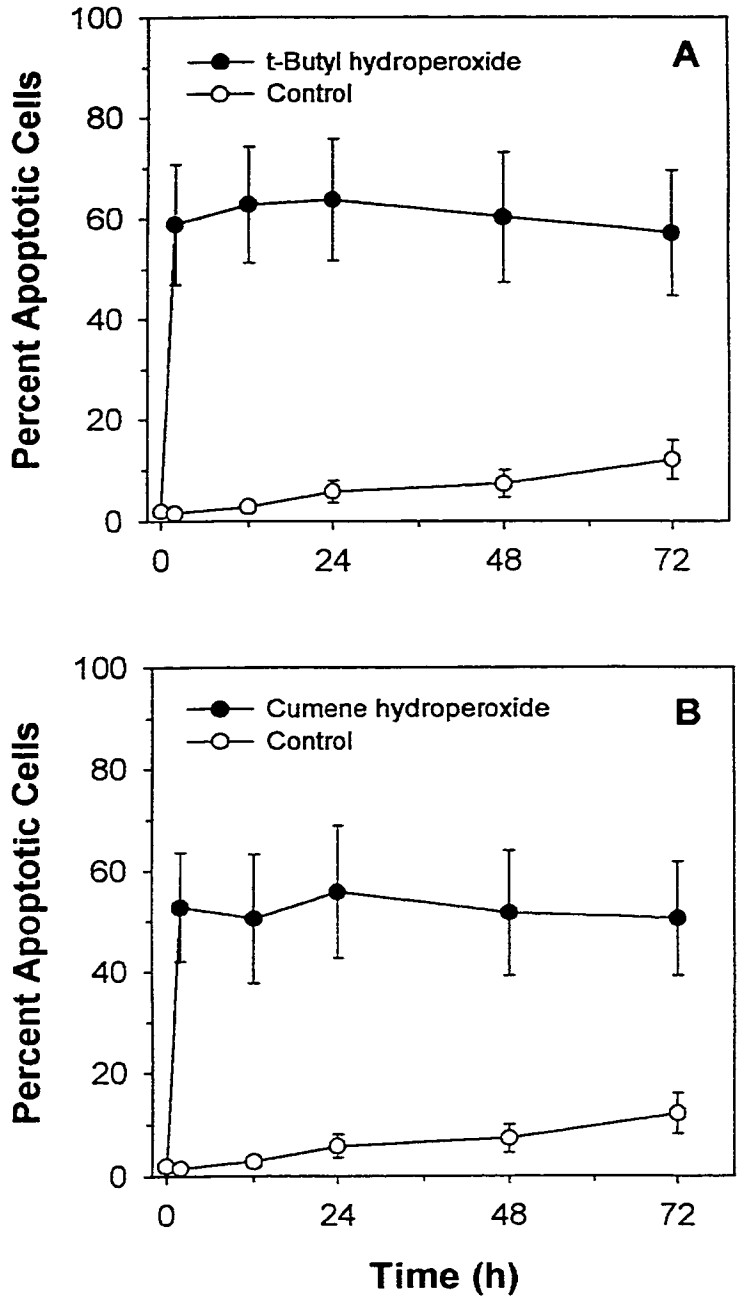

Figure 1.7. Time Course for the Appearance of Apoptotic Cells Following Exposure to Membrane Oxidizing Agents. Lymphocytes were treated with 400μM t-butyl hydroperoxide (A) or 400μM cumene hydroperoxide (B) for 30min at 37°C and the fraction of apoptotic cells was measured at the indicated times using the comet assay (n=6).

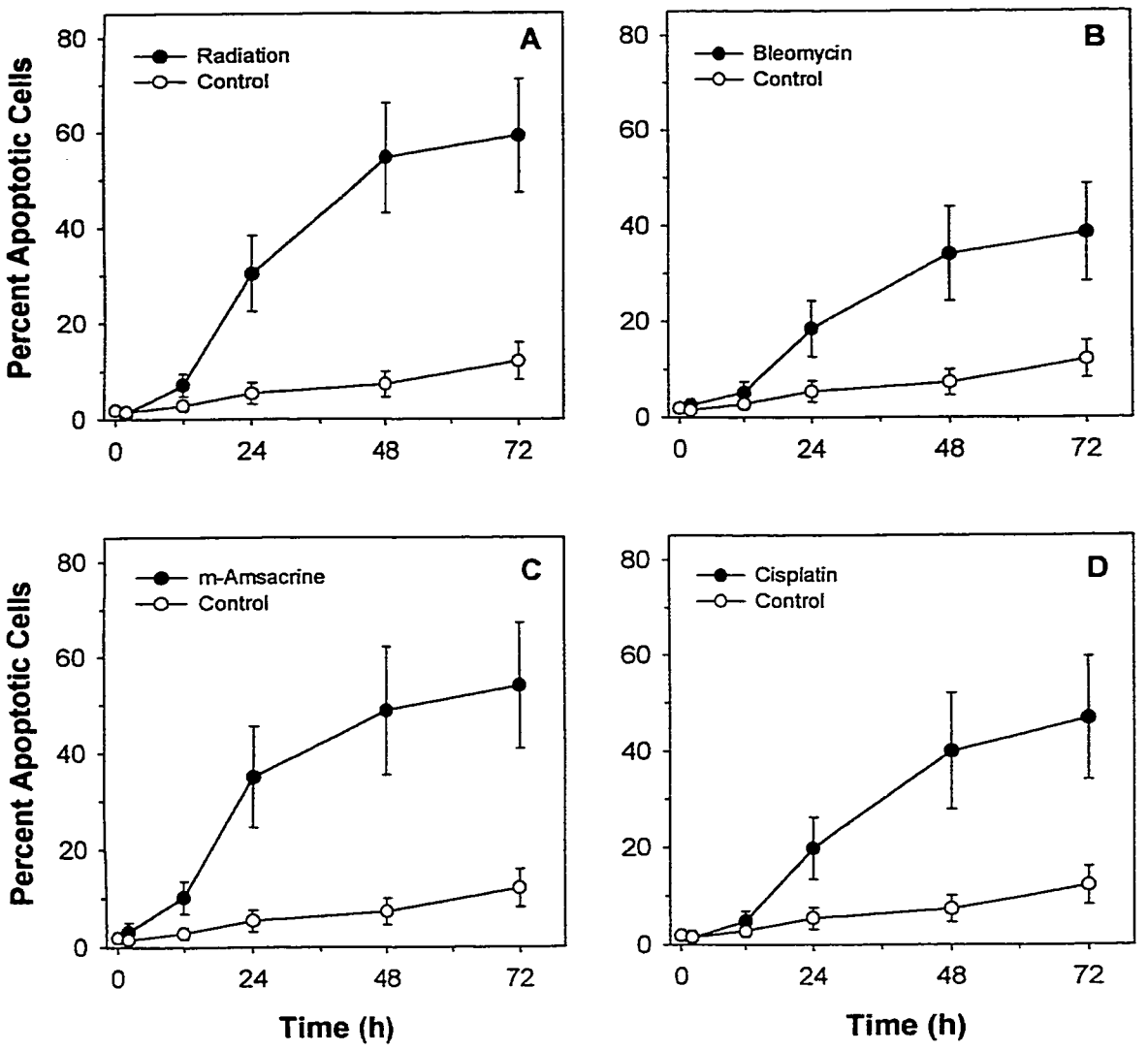

Figure 1.8. Time Course for the Appearance of Apoptotic Cells Following Exposure to DNA Damaging Agents. Lymphocytes were exposed to 4 Gy γ-radiation (A), 100μg/ml bleomycin (B), 80μM m-amsacrine (C), or 60μg/ml cisplatin (D) at 37°C and the fraction of apoptotic cells was determined at the indicated times using the comet assay (n=6).

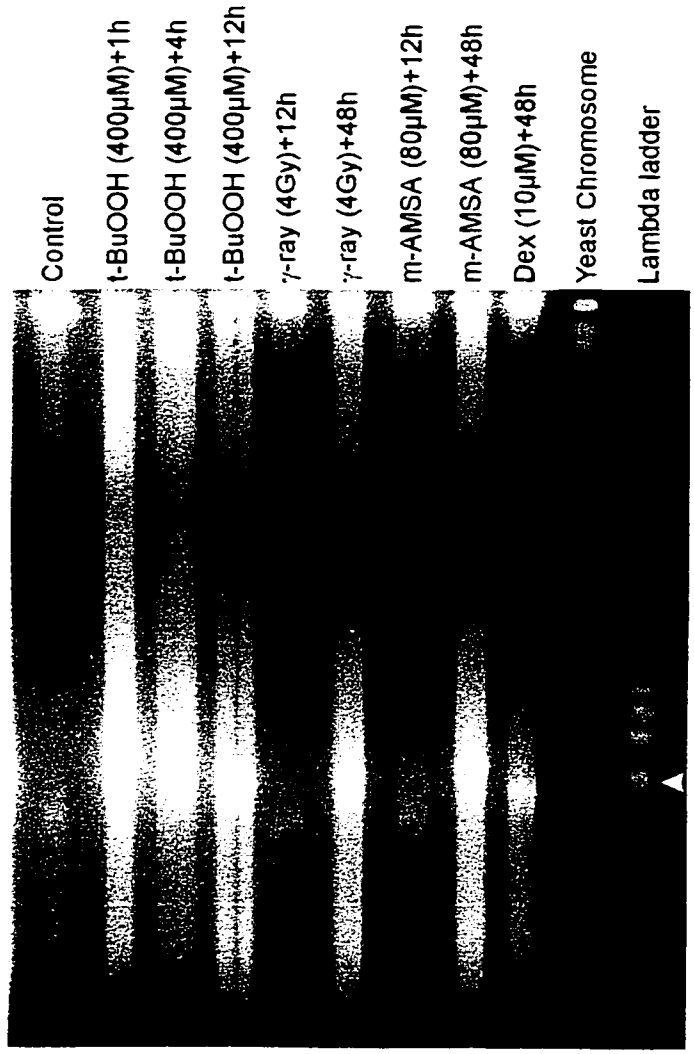

Figure 1.9. PFGE Analysis of Radiation or Drug Induced DNA Fragmentation. DNA fragmentation was analyzed by PFGE in lymphocytes exposed to γ-radiation or the indicated drugs (at 37°C) and incubated for various times at 37°C. The position of the 50kb standard is indicated to the right of the gel (arrowhead).

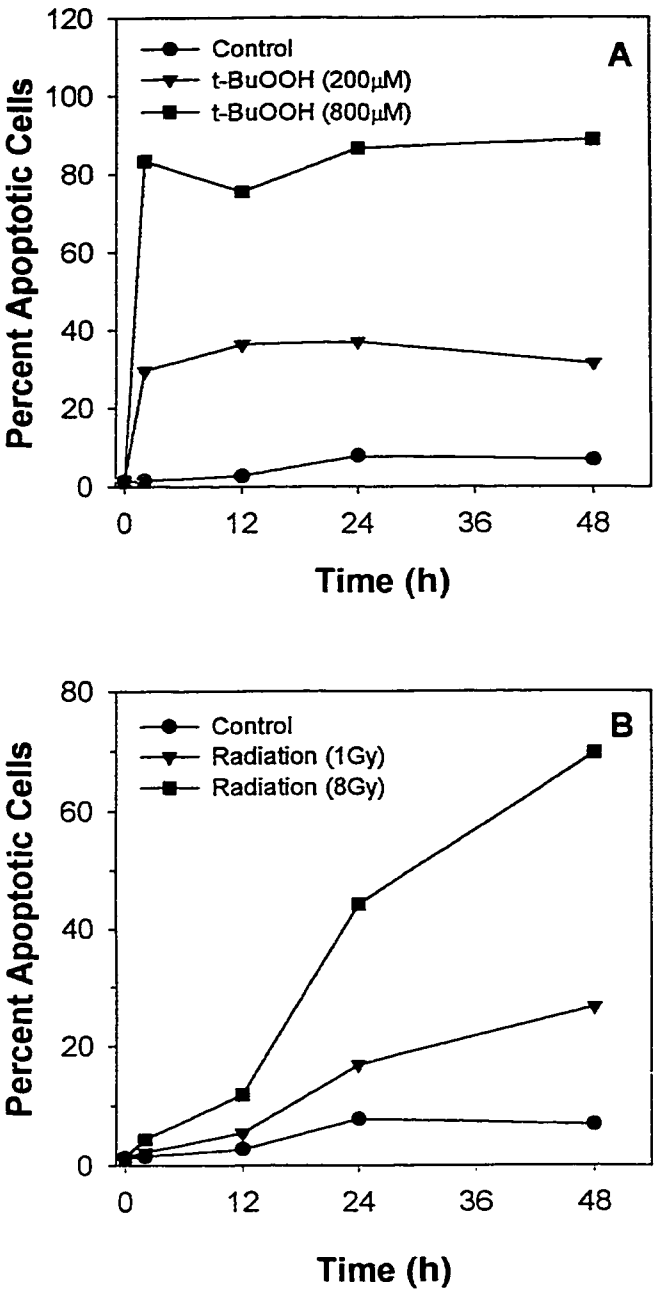

Figure 1.10. Kinetics of t-Butyl Hydroperoxide or Radiation-Induced Apoptosis Following Exposure to Different Drug or Radiation Doses. Lymphocytes were exposed to the indicated concentrations of t-butyl hydroperoxide (A) or different radiation doses (B) and the fraction of apoptotic cells was measured as a function of time after drug or radiation exposure using the comet assay (n=2).

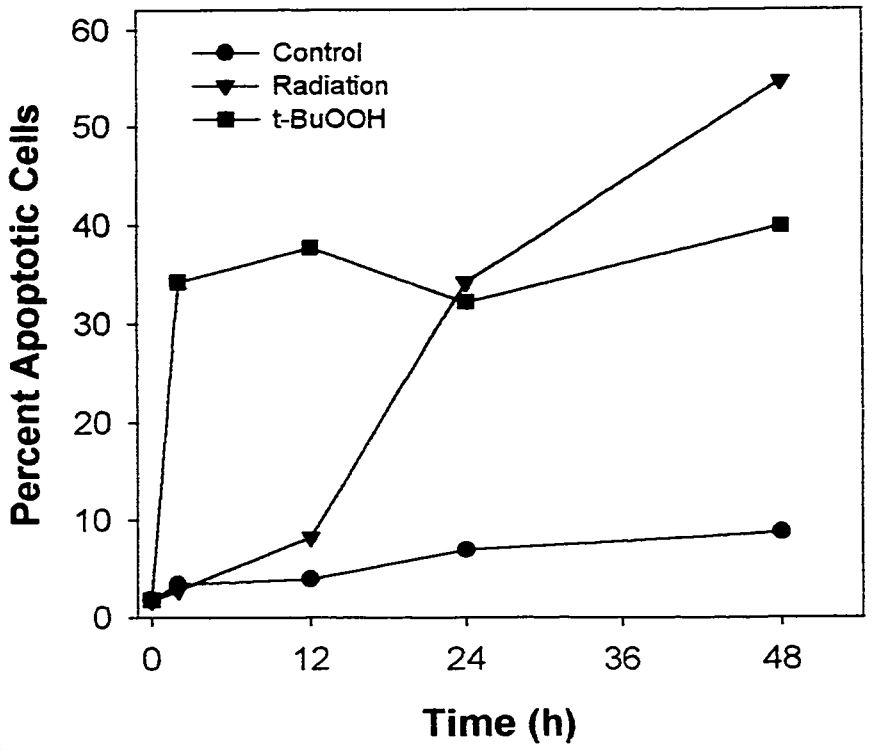

Figure 1.11. Kinetics of t-Butyl Hydroperoxide or Radiation-Induced Apoptosis in Isolated T-lymphocytes. T-cells were exposed to t-butyl hydroperoxide (200μM) or γ-radiation (2 Gy) at 37°C and the fraction of apoptotic cells was determined at the indicated times by the comet assay (n=2).

The Effect of Protein Synthesis Inhibition on Radiation- or t-Butyl Hydroperoxide-Induced Apoptosis

It has previously been reported that in certain cell systems the induction of apoptosis by ionizing radiation requires active protein synthesis and can be blocked by metabolic inhibitors such as cycloheximide or actinomycin D (Sellins and Cohen 1987, Radford and Murphy 1994). Therefore, the effect of cycloheximide on radiation or t-butyl hydroperoxide induced apoptosis was tested to determine whether the different apoptotic kinetics induced by these agents might reflect different requirements for protein synthesis. Different protocols were used to study the effect of cycloheximide on apoptosis induced by these two agents in order to allow for the different kinetics of the apoptotic responses. In one set of samples, lymphocytes were exposed to 4Gy γ-radiation and assayed for apoptosis after a 48 hour incubation in the presence of increasing concentrations of cycloheximide (Figure 1.12A). In a second set of samples, cycloheximide was added 24 hours prior to t-butyl hydroperoxide exposure (200μM, 30 min. at 37°C) and apoptosis was measured after an additional 24 hour incubation (Figure 1.12B). Figure 1.12A shows that when irradiated cells were incubated in the presence of cycloheximide, the extent of radiation-induced apoptosis at 48 hours decreased as a function of cycloheximide concentration. This decrease occurred despite the fact that at higher concentrations cycloheximide itself was found to induce a moderate level of apoptotic cell death. At a cycloheximide concentration of 10μg/ml the extent of radiation-induced apoptosis was found to be significantly reduced from 44.7 ±

9.6 to 9.4 ± 3.7 Q_d units ($p=2.6x10^{-3}$). This result suggests that active protein synthesis is required in the triggering of apoptotic cell death by ionizing radiation. In contrast, Figure 1.12B shows that cycloheximide did not affect the ability of t-butyl hydroperoxide to induce apoptosis. The extent of t-butyl hydroperoxide induced apoptosis in the presence of 10μg/ml cycloheximide (32.4 ± 9.3 Q_d units) was not significantly different ($p=0.14$) than in the absence of cycloheximide (34.8 ± 8.9 Q_d units). This result suggests that the induction of apoptosis by t-butyl hydroperoxide does not require active protein synthesis.

Further experiments were conducted to determine whether radiation-induced apoptosis could occur after removal of cycloheximide. Figure 1.13 shows the development of apoptosis as a function of time after radiation exposure in cells incubated in the presence or absence of cycloheximide (10μg/ml). A third sample was washed 24 hours after radiation exposure to remove cycloheximide and then incubated for an additional 48 hours in cycloheximide-free media. Similar to the results shown in figure 1.12A, the presence of cycloheximide during post-irradiation incubation significantly reduced the level of radiation-induced apoptosis. However, when cycloheximide was removed after 24 hours, lymphocytes proceeded to undergo apoptosis during subsequent incubation in drug-free media, reaching levels similar to irradiated samples incubated in the absence of cycloheximide. This result

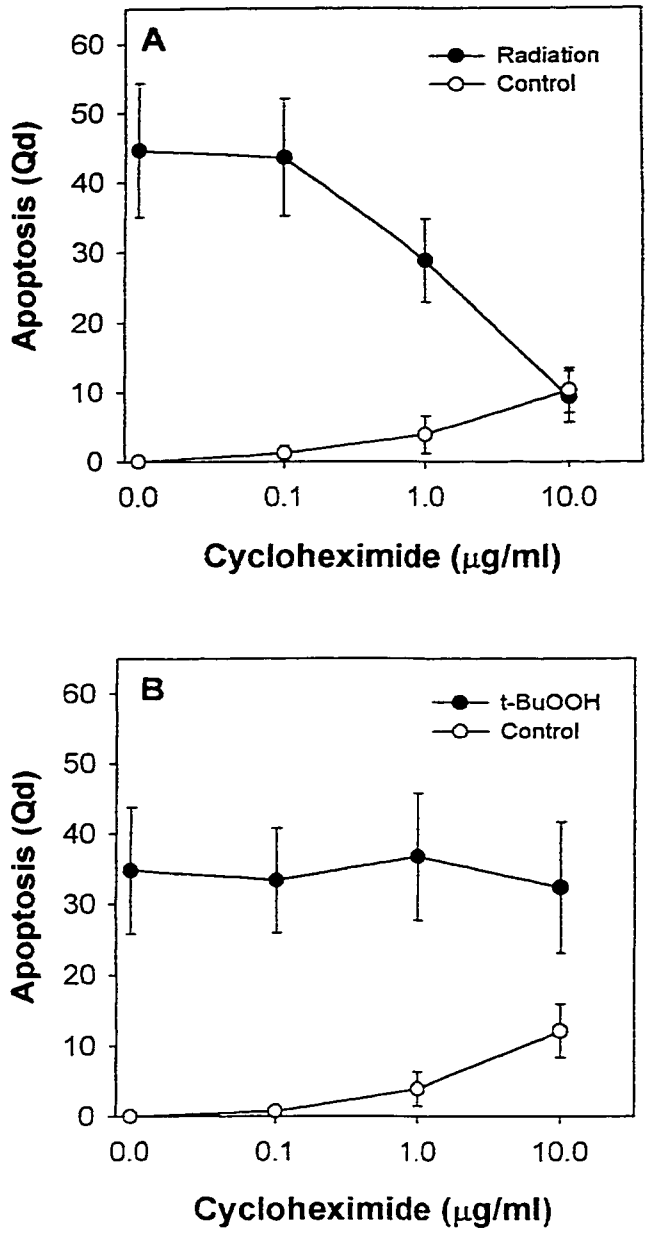

Figure 1.12. The Effect of Cycloheximide on Radiation or t-Butyl Hydroperoxide Induced Apoptosis. Lymphocytes were exposed to 4 Gy γ-radiation (A) or 200μM t-butyl hydroperoxide (B) at 37°C and incubated in the presence of increasing concentrations of cycloheximide. Panel A. Cycloheximide was added 30 minutes prior to irradiation and apoptosis was measured 48 hours later by the FADU assay (n=4). Panel B. Cycloheximide was added 24 hours prior to t-butyl hydroperoxide treatment and apoptosis was measured after an additional 24 hour incubation (n=3).

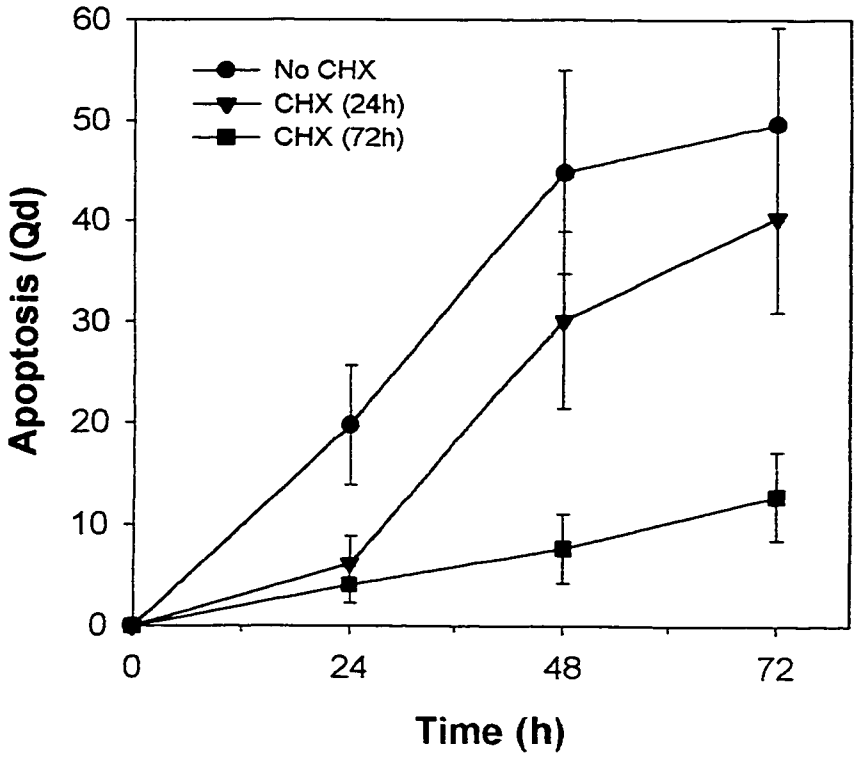

Figure 1.13. The Inhibition of Radiation Induced Apoptosis by Cycloheximide is Reversible. Lymphocytes exposed to 4 Gy γ-radiation were incubated in the presence or absence of cycloheximide (10μg/ml) and apoptosis was measured at the indicated times by the FADU assay. Additionally, a second culture containing cycloheximide was washed after 24 hours to remove the drug and then incubated in drug free media for the remaining 48 hours. Apoptosis induced by cycloheximide in unirradiated controls has been subtracted from corresponding radiation treated values.

suggests that the inhibition of radiation-induced apoptosis by cycloheximide can be reversed upon removal of the protein synthesis block.

Comparison of t-Butyl Hydroperoxide and Ionizing Radiation for the Induction of DNA Damage, Lipid Peroxidation, and Apoptotic Cell Death

To investigate the potential role of oxidative membrane damage in the induction of apoptosis, t-butyl hydroperoxide and γ-radiation were compared for their relative abilities to stimulate lipid peroxidation (Figure 1.14) and apoptotic cell death (Figure 1.15). Figure 1.14 shows that exposure to t-butyl hydroperoxide at 0°C or 37°C resulted in a concentration dependent increase in lipid peroxidation. However, the extent of lipid peroxidation was found to be approximately 4 fold higher following exposure to t-butyl hydroperoxide at 37°C than at 0°C. Figure 1.15 shows that exposure to t-butyl hydroperoxide at 37°C also produced a dose dependent increase in apoptotic cell death. In contrast, when cells were exposed to t-butyl hydroperoxide at 0°C apoptotic cell death was not induced during subsequent incubation at 37°C. At the highest concentration tested (800uM) exposure to t-butyl hydroperoxide at 37°C resulted in an apoptotic frequency of 85.9 ± 11.3% as compared to only 9.0 ± 3.4% in cells exposed to the same concentration at 0°C. In fact, the frequency induced at 0°C was not significantly different than the level of apoptosis observed in untreated control cells, 7.2 ± 2.4% ($p=0.31$).

Despite its ability to induce similar levels of apoptosis as t-butyl hydroperoxide (at 37°C), ionizing radiation was found to be comparatively

ineffective at stimulating lipid peroxidation. The extent of lipid peroxidation caused by exposure to 200μM t-butyl hydroperoxide (at 37°C) was approximately 10-fold higher than that produced by 2Gy γ-radiation (Figure 1.14) despite the fact that these two treatments resulted in a similar level of apoptotic cell death, 36.6 ± 9.7% and 38.4 ± 9.15%, respectively (Figure 1.15). This result indicates a lack of correlation between radiation-induced lipid peroxidation and apoptotic cell death.

Although the cytotoxicity of t-butyl hydroperoxide has been attributed to its ability to cause oxidative damage of cell membranes, other studies have indicated that peroxides can also cause free radical mediated DNA damage, mainly single-strand breaks (Ward 1985, Latour *et al.* 1995). Figure 1.16 shows that a substantial level of DNA damage was produced in lymphocytes exposed to t-butyl hydroperoxide at 0°C. In fact, as measured by the FADU assay, the number of DNA strand breaks produced by t-butyl hydroperoxide reached a level similar to that produced by a 4Gy dose of γ-radiation. However, since exposure to t-butyl hydroperoxide at 0°C did not result in the induction of apoptotic cell death (Figure 1.15), this result suggests that the types of DNA lesions produced by t-butyl hydroperoxide must not be effective at triggering apoptosis.

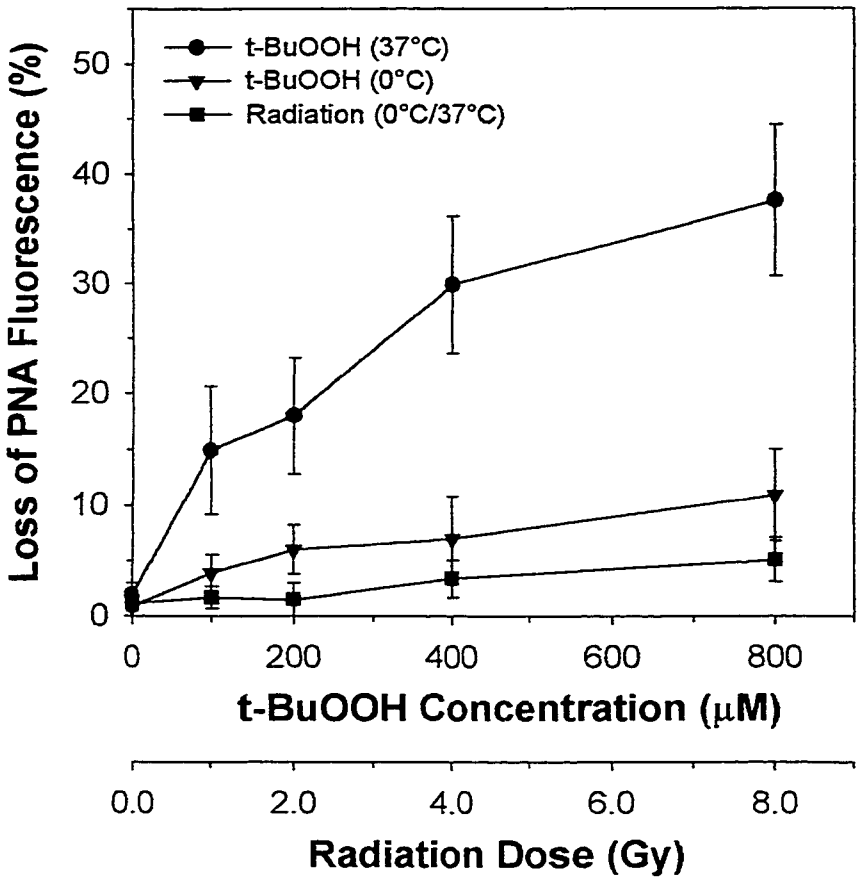

Figure 1.14. Dose Response Comparison for t-Butyl Hydroperoxide and Radiation Induced Lipid Peroxidation. Parinaric acid labelled lymphocytes were exposed to t-butyl hydroperoxide (at 0°C or 37°C) or γ-radiation (at 0°C) and the loss of parinaric acid fluourescence was determined 30 minutes after initiation of drug or radiation exposure. Irradiations were done at 0°C and samples were incubated for 30 minutes at 37°C prior to measurement (n=4).

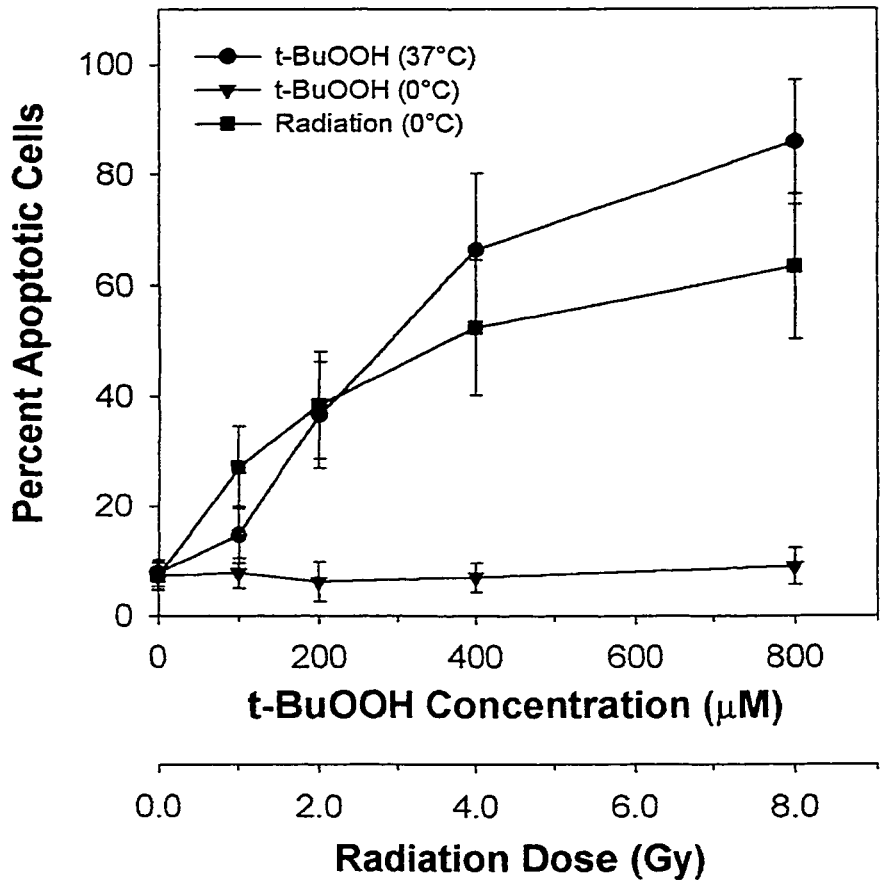

Figure 1.15. Dose Response Comparison for t-Butyl Hydroperoxide and Radiation Induced Apoptosis. The fraction of apoptotic cells was measured 48 hours after exposure to radiation or t-butyl hydroperoxide using the TdT assay (n=4).

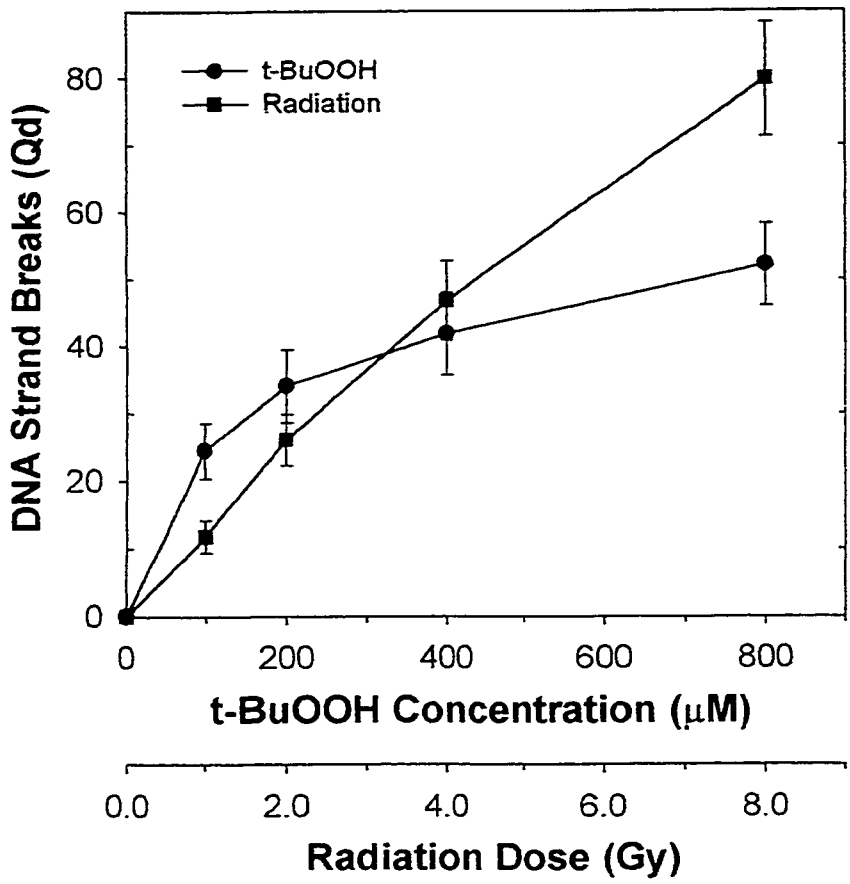

Figure 1.16. Dose Response Comparison for t-Butyl Hydroperoxide and Radiation Induced DNA Damage. Lymphocytes were exposed to radiation or t-butyl hydroperoxide at 0°C and then assayed immediately for DNA damage using the FADU assay (n=5).

The Effect of Free Radical Scavengers on Radiation- or t-Butyl Hydroperoxide-Induced Apoptosis

The role of oxidative membrane damage in the triggering of apoptosis by ionizing radiation or t-butyl hydroperoxide was further evaluated using the lipid soluble free radical scavenger vitamin E. To test the ability of vitamin E to protect against lipid peroxidation in lymphocytes, cells were pretreated with increasing concentrations of vitamin E (for 1 hour at 37°C) and assayed for lipid peroxidation following exposure to 400μM t-butyl hydroperoxide (at 37°C). Figure 1.17A shows that preincubating cells with vitamin E reduced the extent of lipid peroxidation induced by exposure to t-butyl hydroperoxide, although the degree of protection did not appear to increase with vitamin E concentrations above 50μM. Vitamin E at a concentration of 200μM was found to significantly decrease the loss in parinaric acid fluorescence (ie. lipid peroxidation) induced by t-butyl hydroperoxide from 34.1 ± 8.4% to 20.6 ± 7.1% ($p=4.0x10^{-3}$).

In a second set of experiments the effect of vitamin E supplementation on radiation or t-butyl hydroperoxide induced apoptosis was investigated. Lymphocytes preincubated with or without vitamin E (200μM) were exposed to t-butyl hydroperoxide or γ-radiation and then assayed for apoptosis 48 hours later. Figure 1.17B shows that the level of apoptosis induced by t-butyl hydroperoxide (400μM) was significantly reduced from 59.5 ± 11.6 to 44.9 ± 10.4 Q_d units ($p=2.8x10^{-3}$) in cells preincubated with 200μM vitamin E. In contrast, the level of radiation-induced apoptosis was not significantly different in cells preincubated with (Q_d =38.7 ± 9.3) or without (Q_d =41.3 ± 8.3) vitamin E (p=0.17). Figure

1.17B also shows that preincubation with 0.4% ethanol (the equivalent solvent concentration at 200μM vitamin E) did not significantly affect the ability of t-butyl hydroperoxide to stimulate apoptotic cell death ($p=0.48$).

The role of free radical mediated DNA damage in the triggering of apoptosis by ionizing radiation was investigated using the water soluble hydroxyl radical scavenger dimethyl sulfoxide (DMSO). Lymphocytes were exposed to ionizing radiation (4Gy) in the presence of increasing concentrations of DMSO and then assayed either immediately for DNA damage or incubated for 48 hours in DMSO-free media and assayed for apoptosis. Figure 1.18A shows that the presence of DMSO during irradiation results in a concentration dependent decrease in radiation-induced DNA strand breaks and subsequent apoptotic cell death. At a concentration of 1M DMSO the level of radiation-induced DNA strand breaks was reduced by 61.7% ($p=4.3\times10^{-3}$) and the extent of radiation-induced apoptosis was decreased by 39.6% ($p=1.1\times10^{-2}$). In contrast, Figure 1.18B shows that 1M DMSO did not affect the ability of t-butyl hydroperoxide to trigger DNA strand breaks ($p=0.08$) or apoptotic cell death ($p=0.47$). Transient exposure to DMSO (1M) alone did not result in a significant induction of DNA damage ($p=0.40$) or apoptotic cell death ($p=0.43$) compared to untreated controls.

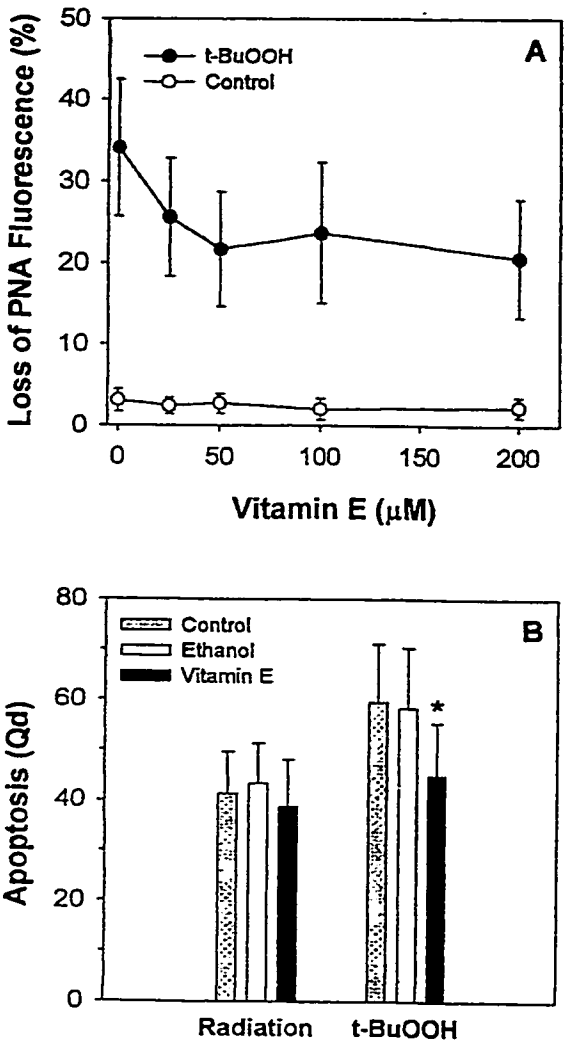

Figure 1.17. The Effect of Vitamin E on t-Butyl Hydroperoxide Induced Lipid Peroxidation and Apoptotic Cell Death. Panel A. The loss of parinaric acid fluorescence was measured following exposure to 400μM t-butyl hydroperoxide (30min, 37°C) in parinaric acid labelled lymphocytes pretreated with increasing concentrations of vitamin E for 1 hour at 37°C (n=4). Panel B. Apoptosis was measured by the FADU assay 48 hours after exposure to 4Gy γ-radiation or 400μM t-butyl hydroperoxide (at 37°C) in lymphocytes preincubated with or without 200μM vitamin E (1h at 37°C) or with 0.4% ethanol as a solvent control (n=4). Asterix indicates that the extent of apoptosis was significantly less ($p<0.05$) in cells pretreated with vitamin E than in nonpretreated cells.

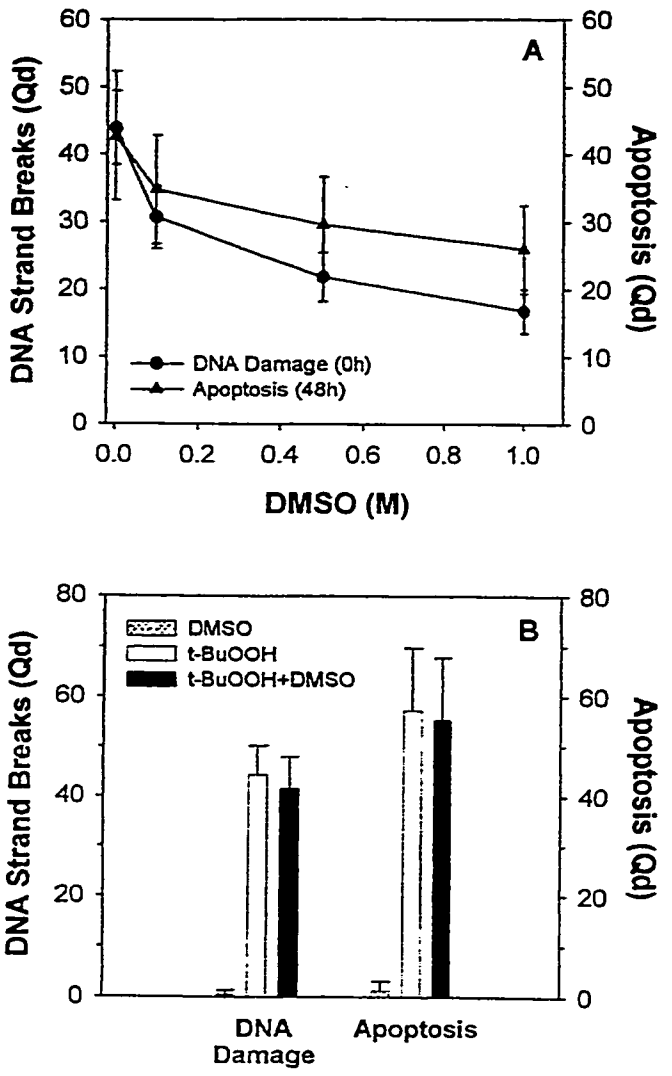

Figure 1.18. The Effect of DMSO on Radiation or t-Butyl Hydroperoxide Induced DNA Damage and Apoptotic Cell Death. Panel A. Lymphocytes were incubated with increasing concentrations of DMSO for 30 minutes prior to and during exposure to 4 Gy γ-radiation at 0°C. Cells were then washed twice with ice-cold media to remove DMSO and assayed either immediately for DNA damage or incubated for 48 hours and assayed for apoptosis using the FADU assay. Panel B. Lymphocytes were exposed to 400μM t-butyl hydroperoxide at 37°C in the presence or absence of 1M DMSO, washed, and then assayed either immediately for DNA damage or incubated for 48 hours and assayed for apoptosis using the FADU assay.

The Effect of DNA Repair Inhibition on Radiation- or t-Butyl Hydroperoxide-Induced Apoptosis

The role of DNA damage in the induction of apoptosis by ionizing radiation was further examined using the DNA repair inhibitor cytosine arabinoside (Ara-C). The ability of Ara-C to inhibit the repair of radiation-induced DNA strand breaks in lymphocytes is demonstrated in Figure 1.19A. The figure shows that while approximately 85% of the DNA strand breaks produced by a 4Gy dose of γ-radiation were removed during a 2 hour incubation at 37°C, less than 20% were removed when Ara-C (10μM) was present during this period. The level of DNA strand breaks remaining 2 hours after radiation exposure was found to be significantly greater in cells incubated in the presence of Ara-C, 34.8 $\pm$ 6.4 Qd units, than in the absence of Ara-C, 6.6 $\pm$ 1.8 Qd units ($p=5.4 \times 10^{-3}$). A similar result was observed when Ara-C was added to cells treated with t-butyl hydroperoxide at 0°C (Figure 1.19B). Cells subsequently allowed to repair for 2 hours at 37°C removed approximately 95% of the DNA strand breaks produced by t-butyl hydroperoxide, whereas incubation in the presence of Ara-C resulted in the repair of less than 20% of these lesions. The level of residual DNA strand breaks present 2 hours after exposure to t-butyl hydroperoxide was found to be significantly greater in cells incubated in the presence of Ara-C, 41.5 $\pm$ 8.4, than in the absence of Ara-C, 2.3 $\pm$ 1.2 ($p=1.9 \times 10^{-2}$). The figure also shows that unirradiated control cells incubated with Ara-C accumulated a small amount of

DNA strand breaks presumably as a result of the inhibition of repair of spontaneously produced DNA lesions.

The effect of DNA repair inhibition on radiation-induced apoptosis is shown in Figure 1.20A. Ara-C mediated DNA repair inhibition resulted in an approximately 2-fold increase in the fraction of apoptotic cells (22.5 ± 5.9% vs 44.9 ± 8.3%) present 48 hours after radiation exposure. Although, incubation with Ara-C alone was found to result in a small induction of apoptosis (3.9 ± 1.5%), the increase in radiation-induced apoptosis observed in repair inhibited cells was found to be significantly greater than the combined level of apoptosis induced by exposure to radiation or Ara-C alone ($p=1.23x10^{-2}$). The kinetics of radiation-induced apoptosis were similar in the presence or absence of Ara-C with the frequency of apoptotic cells beginning to rise approximately 12 hours after irradiation and increasing up to at least 48 hours.

T-butyl hydroperoxide exposures at 0°C could produce high levels of DNA strand breaks (Figure 1.16) and, this damage was apparently ineffective at triggering apoptosis (Figure 1.15). However, Figure 1.20B shows that when cells were exposed to t-butyl hydroperoxide (at 0°C) and incubated with Ara-C (2 hours at 37°C) a significant induction of apoptotic cell death was apparent 48 hours after drug exposure ($p=1.2x10^{-2}$). Furthermore, the induction of apoptosis proceeded with slow kinetics similar to that of ionizing radiation and other DNA damaging agents (Figure 1.8) and not like the rapid apoptotic kinetics observed following exposure to t-butyl hydroperoxide at 37°C (Figure 1.7).

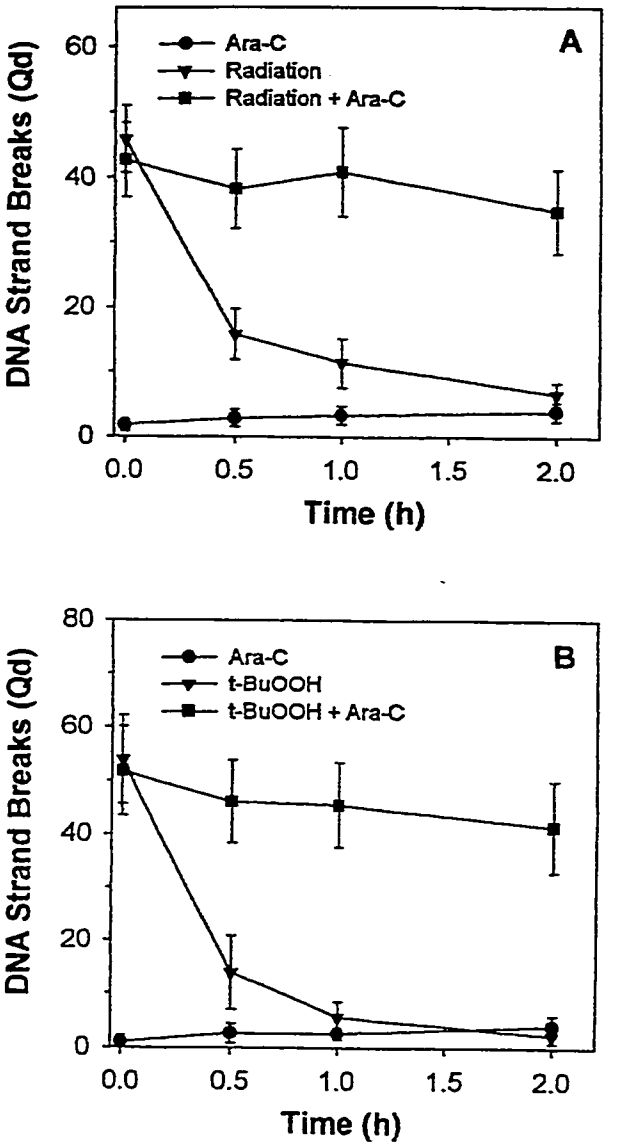

Figure 1.19. The Effect of Ara-C on the Repair of Radiation or t-Butyl Hydroperoxide Induced DNA Strand Breaks. Lymphocytes were exposed to 4Gy γ-radiation (A) or 800μM t-butyl hydroperoxide (B) at 0°C and allowed to repair at 37°C in the presence or absence of 10μM Ara-C. DNA damage was measured at the indicated times using the FADU assay.

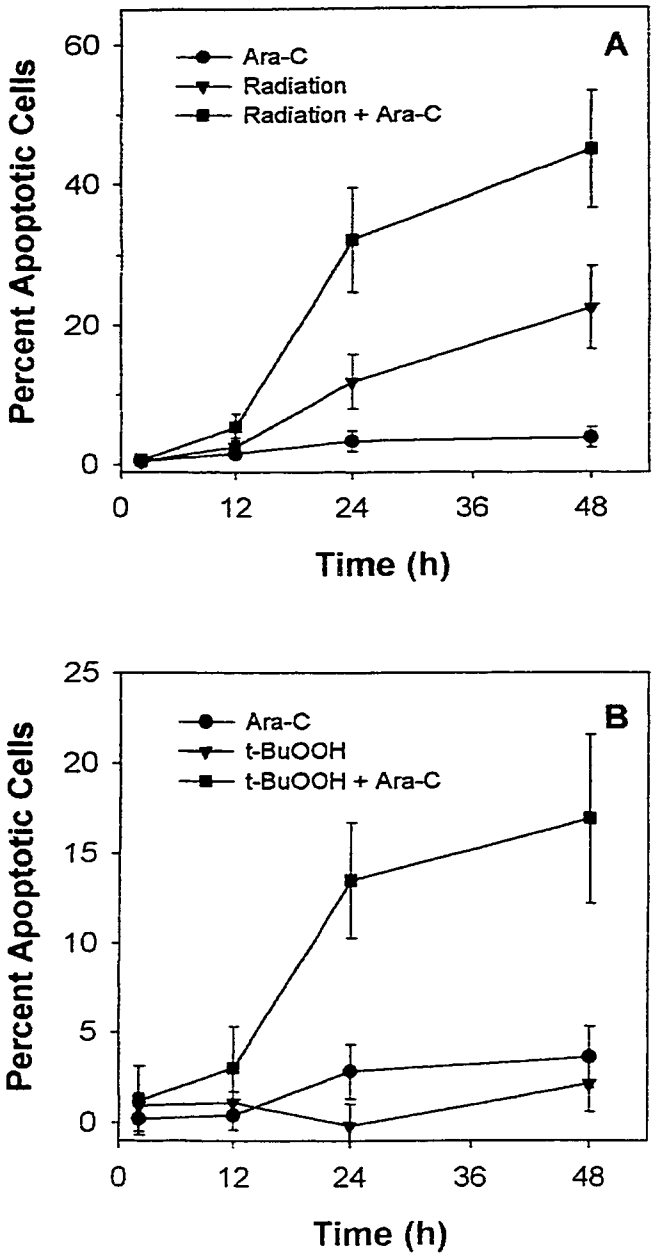

Figure 1.20. The Effect of DNA Repair Inhibition on Radiation or t-Butyl Hydroperoxide Induced Apoptosis. Lymphocytes were exposed to 1Gy γ-radiation (A) or 800μM t-butyl hydroperoxide (B) at 0°C and incubated for 2 hours at 37°C in the presence or absence of 10μM Ara-C. After washing to remove unincorporated Ara-C, cells were resuspended in drug-free media and assayed for apoptosis at the indicated times using the comet assay (n=4).

DISCUSSION

Membrane Oxidizing Agents and DNA Damaging Agents Trigger Apoptosis by Distinct Pathways

The experiments presented here were directed toward resolving the conflicting views presented in the literature concerning the identity of the cellular targets involved in radiation-induced apoptotic cell death. Our experiments show that apoptosis could be induced in human lymphocytes by the DNA damaging agents bleomycin, cisplatin, and m-amsacrine. Lymphocytes were also shown to be sensitive to the induction of apoptosis by the membrane oxidizing agents t-butyl hydroperoxide and cumene hydroperoxide. All of these agents were shown to induce concentration dependent increases in the frequency of cells exhibiting chromatin condensation and DNA fragmentation consistent with apoptosis (Figures 1.5 and 1.6). Furthermore, when analyzed by PFGE these agents were shown to produce a pattern of DNA fragmentation (Figure 1.9) considered characteristic of apoptotic cell death (Oberhammer *et al.* 1993, Cohen *et al.* 1994, Walker *et al.* 1994).

The membrane oxidizing agents and DNA damaging agents, however, were shown to trigger apoptosis by kinetically distinct pathways. The lipophilic oxidizing agents, t-butyl hydroperoxide and cumene hydroperoxide, triggered a rapid induction of the apoptotic process with the frequency of apoptotic cells reaching maximal levels within 2 hours of drug exposure (Figure 1.7). In contrast, exposure to the DNA damaging agents bleomycin, cisplatin, or m-

amsacrine resulted in a delayed triggering of the apoptotic response. The fraction of apoptotic cells increased only slightly within the first 12 hours after drug exposure and did not reach maximal levels until approximately 48-72 hours post-treatment (Figure 1.8). Consistent with its DNA damaging properties, ionizing radiation was shown to trigger apoptosis with slow kinetics similar to that of the various DNA damaging agents (Figure 1.8).

The kinetics of apoptotic cell death was measured in these studies using the comet assay which detects apoptosis associated HMW DNA fragmentation in individual cells (Olive *et al.* 1995). This endonucleolytic cleavage of DNA into specific HMW fragments is considered to be an early and indispensible event in the apoptotic process (Brown *et al.* 1993, Oberhammer *et al.* 1993, Cohen *et al.* 1994, Walker *et al.* 1994). Therefore, the different apoptotic kinetics induced by the DNA damaging agents and membrane oxidizing agents reflects differences in the rates of initiation of the apoptotic process and not differences in the rate of progression through the apoptotic process itself. Furthermore, it was shown that the different apoptotic kinetics induced by these agents did not result from a dose related change in the kinetics of the apoptotic response (Figure 1.10). nor from the response of different cell populations (Figure 1.11). These results suggest that apoptosis can be triggered in human lymphocytes by two kinetically distinct mechanisms: a rapid induction pathway triggered by lipophilic oxidizing agents, and a slow induction pathway triggered by DNA damaging agents.

It has previously been reported that in certain cell systems the induction of apoptosis by ionizing radiation requires active protein synthesis and can be

blocked by metabolic inhibitors such as cycloheximide (Sellins and Cohen 1987, Radford and Murphy 1994). Here it was shown that in human lymphocytes cycloheximide could inhibit radiation but not t-butyl hydroperoxide induced apoptosis (Figure 1.12), further indicating that these agents are triggering apoptosis through different pathways. The ability of cycloheximide to inhibit radiation-induced apoptosis indicates that active protein synthesis is required in this process. A similar dependency for active protein synthesis in the induction of apoptosis has been reported in murine lymphocytes exposed to ionizing radiation (Sellins and Cohen 1987), m-amsacrine (Walker *et al.* 1991), or cisplatin (Sorenson *et al.* 1990). Another report, however, has indicated that while cycloheximide could block radiation-induced apoptosis it had no effect on heat induced apoptosis in the same cells (Sellins and Cohen 1991). The data here indicate that the newly synthesized protein(s) are likely to be involved in the triggering mechanism of the slow apoptotic process and not part of the apoptotic machinery itself since inhibition of protein synthesis did not block the apoptotic process in t-butyl hydroperoxide treated cells. Similar to previous reports in other cell systems (Vedekis and Bradshaw 1983, Martin *et al.* 1990), however, prolonged exposure to cycloheximide itself resulted in a modest induction of apoptotic cell death. This may result from either the cells inability to synthesize essential proteins, its inability to synthesize proteins which normally inhibit apoptosis, or inhibition of synthesis of repair enzymes required for repair of spontaneously occurring DNA lesions.

Membrane Oxidation as a Trigger for Apoptosis

It has previously been demonstrated that apoptosis can be induced in different cell systems by lipophilic oxidizing agents such as t-butyl hydroperoxide (Langley *et al.* 1993, Zhong *et al.* 1993, Mukharjee *et al.* 1995) and fatty acid hydroperoxides (Sandstrom *et al.* 1994). It is generally thought that the cytotoxic effects of organic hydroperoxides is mediated by peroxidative damage of cell membranes (Farber *et al.* 1990, Bartoli *et al.* 1994). It is proposed that organic hydroperoxides like t-butyl hydroperoxide (t-BuOOH) are converted to highly reactive alkoxyl radicals (e.g. t-BuO•) in a reaction catalyzed by redox active metal ions (predominately copper or iron). The highly reactive t-BuO• is then proposed to initiate lipid peroxidation by abstracting a hydrogen atom from an (oxidation sensitive) unsaturated fatty acid (LH) in the cell membrane. Subsequent chain propogation reactions produce lipid hydroperoxides (LOOH) resulting in perturbations of membrane structure and function leading to cell death (Girotti 1985, Massa and Giulivi 1993).

$$\text{t-BuOOH} + Fe^{2+} \rightarrow \text{t-BuO}\bullet + Fe^{3+}$$

$$\text{t-BuO}\bullet + \text{LH} \rightarrow \text{t-BuOH} + \text{L}\bullet$$

$$\text{L}\bullet + O_2 \rightarrow \text{LOO}\bullet$$

$$\text{LOO}\bullet + \text{LH} \rightarrow \text{LOOH} + \text{L}\bullet$$

This model has been supported by the finding that preincubating cells with the iron chelating agent, desferrioxamine, or the lipophilic antioxidant vitamin E could

simultaneously protect against t-butyl hydroperoxide stimulated lipid peroxidation and subsequent cell death in hepatocytes (Masaki *et al.* 1989) and L1210 murine leukemia cells (Geiger *et al.* 1993). However, the mode of cell death was not determined in these studies and therefore it is not clear whether a similar mechanism is involved in hydroperoxide induced apoptotic cell death.

In the present study it was demonstrated that the lipophilic oxidizing agents t-butyl hydroperoxide or cumene hydroperoxide could trigger apoptosis through a mechanism kinetically distinct from that induced by known DNA damaging agents. Furthermore, the results presented here suggest that these agents act through a mechanism initiated by membrane oxidative damage. T-butyl hydroperoxide was shown to stimulate both lipid peroxidation (Figure 1.14) and apoptotic cell death (Figure 1.15) in a similar concentration and temperature dependent manner. It was also shown that the lipophilic antioxidant vitamin E could simultaneously protect against t-butyl hydroperoxide induced lipid peroxidation (Figure 1.17A) and subsequent apoptotic cell death (Figure 1.17B).

In addition to its ability to cause lipid peroxidation, t-butyl hydroperoxide was also found to cause significant levels of DNA strand break damage (Figure 1.16). However, this damage appeared to be inconsequential since drug exposures at 0°C did not trigger apoptotic cell death (Figure 1.15). It should be noted, however, that exposure to t-butyl hydroperoxide at 0°C still produced significant membrane oxidation (Figure 1.14) suggesting that any signal for the fast apoptotic process which originates from membrane oxidation must be transient.

The mechanisms by which membrane oxidation events might result in stimulation of the apoptotic process have not been established. It has been suggested that lipid hydroperoxides can cluster in membranes creating pores through which ions or other solutes can diffuse into the cell (Girotti 1985). In fact the disruption of the selective transport of ions across the plasma membrane has been shown to trigger apoptosis. For example, Ca^{2+} ionophores such as A23187 have been shown to stimulate apoptosis in thymocytes (McConkey *et al.* 1989) and the deregulation of Ca^{2+} homeostasis does appear to be a common theme in apoptotic cell death (Marks 1997). It is unclear, however, whether Ca^{2+} functions as a second messenger in signal transduction pathways which activate the apoptotic process or whether it is directly involved in the apoptotic process itself - for example, in the activation of the Ca^{2+}/Mg^{2+}-dependent endonucleases involved in apoptotic DNA fragmentation (Walker *et al.*1994).

A second possible mechanism is that membrane oxidation could result in the activation of redox sensitive signal transduction enzymes located in the plasma membrane. For example, it has been shown that membrane oxidation generated by photodynamic activation of a membrane specific photosensitizer can trigger apoptosis in mouse L5178Y lymphoma cells (Agarwal *et al.* 1991). Like t-butyl hydroperoxide, photodynamic treatment triggered a rapid induction of cell death (within one hour) which did not require active protein synthesis. This induction of apoptosis was associated with a rapid activation of phospholipase C in the plasma membrane with subsequent breakdown of membrane phosphoinositides and rapid release of intracellular Ca^{2+} (Agarwal *et al.* 1993).

An inhibitor of phospholipase C was shown to block these second messenger events as well as subsequent apoptotic DNA fragmentation indicating that apoptotsis was triggered as a result of phospholipase C activation. Interestingly, t-butyl hydroperoxide has been reported to stimulate phospholipase activity in p815 cells (Kondakova *et al.*1995).

Triggering of Apoptosis by Ionizing Radiation and other DNA Damaging Agents

Ionizing radiation and lipophilic oxidizing agents were shown to trigger apoptosis through distinct mechanisms. In contrast to the rapid apoptotic response induced by various lipophilic oxidizing agents, radiation-induced apoptosis was triggered by a process exhibiting slow kinetics and requiring active protein synthesis. The kinetics of this response was similar to that observed for other known DNA damaging agents including cisplatin, m-amsacrine, and bleomycin (Figure 1.8). This radiation-induced apoptotic cell death in human lymphocytes did not correlate with membrane oxidative damage. Ionizing radiation was found to be much less effective than t-butyl hydroperoxide at stimulating lipid peroxidation at doses at which these agents produced similar levels of apoptosis (Figures 1.14 and 1.15). Furthermore, it was shown that the lipophilic antioxidant vitamin E could not protect against radiation-induced apoptosis at concentrations at which it significantly reduced t-butyl hydroperoxide induced lipid peroxidation and apoptotic cell death (Figure 1.17). In another study, however, it was shown that Trolox, a water soluble analogue of vitamin E, could protect against radiation-induced lipid peroxidation as well as apoptotic cell

death in mouse thymocytes (Ramakrishnan *et al.* 1993). However, it is possible that Trolox, being a water soluble free radical scavenger, may also have protected against radiation-induced DNA damage.

On the other hand, the hydroxyl radical scavenger DMSO was shown to protect against radiation-induced DNA strand break damage and subsequent apoptotic cell death in a similar concentration dependent manner (Figure 1.18A). DMSO at the highest concentration tested (1M) did not protect against t-butyl hydroperoxide induced DNA damage or apoptotic cell death (Figure 1.18B). This was not unexpected, however, since the free radical chemistry of t-butyl hydroperoxide does not predict the involvement of hydroxyl radicals (Massa and Giulivi 1993). On the other hand, the lack of an effect of DMSO on t-butyl hydroperoxide induced apoptosis argues that the protective effect on radiation-induced apoptosis is consistent with its free radical scavenging capacity and not some inhibitory effect on the apoptotic process itself. Although it is possible that DMSO might provide a low level of protection against radiation-induced membrane damage, the inability of vitamin E to protect against radiation-induced apoptosis suggests that this would be inconsequential.

The DNA repair inhibitor Ara-C was shown to inhibit the repair of radiation-induced DNA strand breaks (Figure 1.19A) and this repair inhibition enhanced the level of radiation-induced apoptotic cell death (Figure 1.20A). Radiation-induced apoptosis showed similar kinetics in the presence or absence of Ara-C indicating that repair inhibiton was enhancing the slow kinetic pathway. This

result is consistent with a role of DNA damage in the triggering of the kinetically slow apoptotic process by ionizing radiation.

There are at least two possible mechanisms by which Ara-C mediated DNA repair inhibition may lead to potentiation of radiation-induced apoptosis. It is possible that apoptotic signals are not generated by specific types of DNA lesions, but that signalling may depend on the relative persistence of DNA damage. Therefore, lesions such as single-strand breaks may normally be ineffective because they are rapidly repaired. However, inhibiting the repair of single strand breaks would result in the ability of such lesions to generate apoptotic signals. A second possibility is that only certain complex lesions result in an apoptotic signal. Repair inhibition may lead to the formation of more complex DNA lesions (e.g. double strand breaks). Incorporation of Ara-C during repair resynthesis could inhibit the closure of excision repair generated "gaps". The stabilization of such repair "gaps" would increase the likelihood of interaction between closely opposed DNA lesions and could enhance the yield of DNA double-strand breaks (Hiss and Preston 1977, Ahnstrom and Bryant 1982, Iliakis and Bryant 1983). This increase in the level of DNA double strand breaks may then be responsible for the enhanced induction of apoptotic cell death.

Apoptotic cell death from ionizing radiation exposure was inhibited by the protein synthesis inhibitor cycloheximide (Figure 1.12A) but this inhibition could be reversed upon removal of the drug up to at least 24 hours after radiation exposure (Figure 1.13). This suggests that radiation produces a stable signal which, upon release from the protein synthesis block, can still trigger the

apoptotic process. Furthermore, it was shown that apoptotic cell death was initiated in irradiated lymphocytes well after the majority of radiation-induced DNA lesions had been repaired (Figure 1.1). This suggests that the stable apoptotic signal produced by ionizing radiation is likely to be either a persistent DNA lesion (ie. an irrepairable or slowly repiared DNA lesion) or some type of stable secondary signal generated in response to the initial repairable DNA damage. However, if apoptotic signals are produced in response to repairable DNA damage then clearly not all types of repairable damage are effective. Treatment with t-butyl hydroperoxide (at 0°C) was shown to produce significant levels of DNA strand breaks which were rapidly repaired (Figure 1.19B) but ineffective at triggering apoptosis (Figure 1.20B). However, treatment of cells with t-butyl hydroperoxide (at 0°C) to generate DNA strand breaks followed by incubation at 37°C in the presence of Ara-C slowed the repair of DNA strand breaks and initiated the kinetically slow apoptotic process as seen with radiation or other DNA damaging agents. These results are consistent with generation of the apoptotic inducing signal by slowly repaired DNA lesions. However, unrepairable DNA lesions cannot be ruled out since it is not clear whether the repair inhibition is completely reversible.

Ionizing radiation is known to produce a variety of different types of DNA lesions including single-strand breaks, double-strand breaks, and interstrand crosslinks (*reviewed in* Ward 1988). The ability of m-amsacrine or cisplatin to trigger apoptotic cell death suggests that DNA double-strand breaks or interstrand crosslinks can signal the apoptotic response. In contrast, exposure to

t-butyl hydroperoxide (at 0°C) which is known to produce (in addition to membrane oxidation) predominately single-strand breaks (Latour *et al.* 1995) did not trigger apoptosis, suggesting that single strand breaks are not effective signals for the induction of apoptosis. A similar inability of peroxide induced single strand breaks to trigger apoptosis has been reported elsewhere (Radford 1991). On the other hand, a number of studies have demonstrated a correlation between radiation-induced DNA double strand breaks and the induction of apoptotic cell death (Radford 1991, Radford and Murphy 1994, Story *et al.* 1994). Furthermore, some recent studies have demonstrated a relationship between DNA repair capacity and sensitivity to apoptotic cell death. In one such study it was shown that radiation-induced apoptosis was markedly increased in a radiation sensitive mutant CHO cell line (Hu and Hill 1996). This cell line is known to have a defect in the DNA binding subunit of the DNA-PK complex and shows higher levels of unrepaired DNA strand breaks following irradiation than the parental cell line. Similarly, cell lines derived from severe combined immunodeficient (scid) mice, which have a defect in the catalytic subunit of DNA-PK (Araki *et al.* 1997), have been shown to be extremely sensitive to the induction of apoptosis by radiation as compared with the parental line (Meng *et al.* 1998, Radford and Aldridge 1999). This defect has been reported to result in a decreased level of double strand break rejoining (Biederman *et al.* 1991) or delayed but complete rejoining (Nevaldine *et al.* 1997).

In summary, the results presented here demonstrate that apoptosis can be induced in human lymphocytes by two distinct pathways. Membrane

oxidizing agents trigger apoptosis through a rapid induction pathway that does not require active protein synthesis and which may be initiated by membrane oxidative processes that produce a transient signal. Ionizing radiation and the other DNA damaging agents, on the other hand, trigger apoptosis by a slow induction pathway which requires active protein synthesis and that is initiated by a persistent signal related to slowly repaired or unreparable DNA damage.

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CHAPTER 2

APOPTOSIS AND THE ADAPTIVE RESPONSE TO IONIZING RADIATION

INTRODUCTION

The detrimental effects of radiation exposure have typically been attributed to its ability to cause DNA damage (Obe *et al.* 1992). Fortunately, cells possess complex and efficient DNA repair processes capable of recognizing and repairing most genomic damage (reviewed in Sancar and Sancar 1988). These repair processes, however, are not perfect and occasionally damaged DNA is misrepaired or incompletely repaired. In certain cell types, such as resting lymphocytes, the persistence of DNA damage can result in the triggering of apoptotic cell death (Cregan *et al.* 1999). It has been proposed that the induction of apoptosis in response to DNA damage functions as a protective mechanism by eliminating genetically damaged cells (Kerr *et al.* 1994, Kondo 1998). Cells containing misrepaired DNA damage which are not eliminated by cell death could represent a potential risk for carcinogenesis. Thus, the enhancement of either an error-free DNA repair pathway or apoptotic cell death could potentially reduce the carcinogenic risk of radiation exposure. Since both DNA repair and apoptosis are regulated genetic programs, it might be expected that these processes could be modified by alterations in gene expression or changes in the activity of signal transduction pathways. Both processes have been shown to be affected by low doses of ionizing radiation (Wolff *et al.* 1989, Woloschak *et al.*

1990a; 1990b, Weichelsbaum *et al.* 1991, Azzam *et al.* 1992, 1994, Baxter *et al.* 1992, Ikushima *et al.* 1992, Meyers *et al.* 1992, 1995).

The term "adaptive response" can be defined as the induction of a protective mechanism which confers resistance to the detrimental effects of radiation exposure. The term was originally used to describe a phenomenon in which pre-exposure of cells to a low dose (ie. "adapting" dose) of radiation rendered cells less susceptible to chromosomal damage induced by a subsequent high dose (ie. "challenge" dose) of radiation. It has been proposed that an error-free DNA repair process is induced by the low dose radiation exposure such that adapted cells also show a reduction in the frequency of mutation (Sanderson and Morley 1986, Kelsey *et al.* 1991, Rigaud *et al.* 1993) and neoplastic transformation induced by a subsequent exposure (Azzam *et al.* 1994) as well as a reduction in spontaneous neoplastic transformation (Azzam *et al.* 1996). The adaptive response has been shown to be induced in mitogen stimulated lymphocytes by low levels of radioactive (tritiated) thymidine (Olivieri *et al.* 1984) or low doses of X-rays or γ-rays (Shadley and Wolff 1987, Farooqi and Kesavan, 1993). Induction of the adaptive response has been found to be dependent upon a number of factors including the adapting dose, dose rate, expression time, culture conditions, and stage of the cell cycle (reviewed in Wolff 1992). In addition to being induced by low doses of ionizing radiation, the adaptive response has been shown to be triggered by low concentrations of the DNA damaging chemicals bleomycin (Vijayalaxmi and Burkart 1989, Wolff *et al.*

1989), cisplatin (Dolling *et al.* 1998), and hydrogen peroxide (Cortes *et al.* 1990, Dominguez *et al.* 1993).

In resting human lymphocytes exposure to ionizing radiation (Payne *et al.* 1992, Delic *et al.* 1993, Hertdvelt *et al.* 1997) or membrane active agents (Sandstrom *et al.* 1994, Cregan *et al.* 1999a) is known to trigger apoptotic cell death. In the present study I tested the effect of exposing unstimulated lymphocytes to a low dose of radiation or a membrane oxidizing agent on the induction of apoptosis by a subsequent exposure to a high dose of ionizing radiation or membrane oxidizing agent. The results show that G_o lymphocytes exposed to an adapting dose of radiation or DNA strand breaking agent can become sensitized to subsequent radiation-induced apoptosis. On the other hand, neither pretreatment affected the level of apoptosis induced by exposure to a high concentration of the membrane oxidizing agent t-butyl hydroperoxide. Since an increase in the elimination of genetically damaged cells by apoptosis could reduce the cancer risk of radiation exposure I suggest that this may represent a novel adaptive response mechanism.

MATERIALS AND METHODS

Cell Preparation and Culture

Human peripheral blood lymphocytes were isolated and cultured as described in Chapter 1.

Irradiations

^{60}Co γ-ray adapting doses were delivered to cells at 37°C at a dose rate of approximately 0.01 Gy/min (Gamma-beam 150, AECL Research). Challenge doses were delivered at a dose rate of approximately 1.0 Gy/min from a ^{60}Co γ-source (Gamma Cell 200, AECL Research). For the challenge exposures, flasks were removed from the 37°C incubator for the time required to deliver the radiation dose (approximately 2 minutes). Control samples were sham irradiated.

Drug Treatments

t-Butyl hydroperoxide was added to cell suspensions (approximately $4x10^6$ cells) at the indicated drug concentrations and samples were incubated for 30 minutes at 0°C in an ice-water bath or at 37°C in a temperature controlled circulating water bath. Control cells were incubated in drug -free media. At the end of the drug exposures, cells were washed twice in ice-cold media to remove unincorporated drug and then cultured as previously described in Chapter 1.

Quantitation of Apoptosis

The level of apoptotic cell death was measured using the FADU and/or TdT assays as previously described in Chapter 1.

Data Analysis

Data points for each treatment type represent the mean and standard error of 3 independently treated replicate samples. Means were compared using students *t*-test and the difference between means was considered statistically significant if $p<0.05$.

RESULTS

Radiation-Induced Adaption to Radiation-Induced Apoptosis

I tested the possibility that pre-exposure of lymphocytes to a low dose of radiation (an "adapting" exposure) could modify the extent of apoptosis induced by a subsequent high dose radiation exposure (a "challenge" exposure). The level of apoptosis induced by a 2Gy challenge dose of radiation was compared in control cells and cells which had been exposed 6 hours earlier to a 0.1Gy adapting dose. Table I shows the results in lymphocytes from 9 different donors, 3 of whom were tested on more than one occassion. In all 15 cases the level of apoptosis induced by the 2Gy challenge exposure was either enhanced or showed no change in cells which had received the adapting dose of radiation. In no case was the level of apoptosis reduced in adapted cells. The extent of the change in sensitivity varied amongst the cells from the different donors, ranging

from no significant change (donors B and D) to a 39.5% increase in donor C (experiment 2). As indicated in Table I, a statistically significant increase was found in 10 of the 15 cases. In these 10 cases the adapting exposure increased the level of apoptosis induced by the challenge exposure by an average of 26.7 ± 5.9%. The results from the donors which showed statistically significant increases in the level of apoptosis as determined by the FADU assay were paralleled by statistically significant increases in the fraction of apoptotic cells measured using the TdT assay. The other donors showed no significant change in either assay.

Cells from three donors (donors A, B, and C) were examined on three different occassions (experiments 4, 5, and 7) to assess the intra-individual variability of this sensitization response. In all 3 experiments involving donor C, the adapting exposure was found to significantly enhance the level of apoptosis induced by a subsequent radiation exposure with relative increases of 39.5% ($p<0.01$), 28.3% ($p<0.05$), and 31.4% ($p<0.05$) respectively. In cells from donor B, the adapting exposure was found to have no significant effect on the level of radiation-induced apoptosis in any of the 3 experiments. In cells from donor A, on the other hand, the adapting exposure was found to induce a significant increase in the level of radiation-induced apoptosis on 2 occasions (25.9% and 28.0%) but a smaller and not statistically significant increase in the other experiment (10.4%).

Lymphocytes from the different individuals exhibited a range in sensitivity to radiation-induced apoptosis. However, there did not appear to be a

correlation between the relative level of radiation-induced apoptosis and the extent of sensitization induced by the adapting dose. For example, the degree of sensitization observed in cells from donors B and G was 4.2% (not significant) and 25.8% ($p<0.05$), respectively, despite the fact that cells from both donors exhibited similar low levels of radiation-induced apoptosis (11.8 and 9.3 Q_d units after the 2Gy exposure, respectively). Likewise, increases of 5.8% (not significant) and 24.9% ($p<0.05$) were observed in cells from donors D and E, respectively, even though these cells exhibited similar high levels of apoptosis (20.8 and 19.7 Qd units respectively).

The effect of varying the time between the adapting and challenge dose exposures on the extent of sensitization is shown in Table II. The level of apoptosis induced by a 2Gy challenge dose of radiation was compared in cells that had received a 0.1Gy adapting dose 0, 6, or 24 hours prior to the challenge exposure. The table shows that in cells from 3 of the 4 donors tested, the level of apoptosis induced by the 2Gy challenge exposure was significantly greater when the adapting dose was given 6 hours prior to the challenge exposure than when it was given immediately before. The cells from these three donors also showed a significant increase when the adapting dose was given 24 hours prior to the challenge exposure (as compared to immediately before). When averaged over the 3 donors, the magnitude of the cellular increase in sensitivity was found to be similar whether the adapting dose was given 6 hours ($26.8 \pm 4.0\%$) or 24 hours ($28.6 \pm 4.7\%$) prior to the challenge exposure. In cells from donor 3 the adapting

Table I. The Effect of Pre-Exposure to Low Dose Radiation on Subsequent Radiation Induced Apoptosis in Human Lymphocytes[a]

Experiment Donor	Adapting Dose (Gy)	Challenge Dose (Gy)	Apoptosis (Qd)[b]	Apoptotic Cells (%)[c]
1 A	-	2.0	18.5±1.8	27.4
	0.1	2.0	23.3±1.6**	35.3*
B	-	2.0	11.8±1.2	17.6
	0.1	2.0	12.3±1.3	18.8
2 C	-	2.0	12.9±1.3	18.1
	0.1	2.0	18.0±1.3**	25.2*
D	-	2.0	20.8±2.1	25.7
	0.1	2.0	22.0±2.4	28.9
3 E	-	2.0	19.7±1.4	24.6
	0.1	2.0	24.6±1.8*	31.4*
F	-	2.0	18.6±1.2	22.8
	0.1	2.0	21.9±1.6*	27.4*
4 B	-	2.0	12.3±1.0	15.9
	0.1	2.0	12.6±1.1	17.2
G	-	2.0	9.3±0.9	18.3
	0.1	2.0	11.7±1.0*	25.2*
5 A	-	2.0	19.2±2.1	26.6
	0.1	2.0	21.2±2.0	29.9
C	-	2.0	18.4±1.7	21.4
	0.1	2.0	23.6±1.8*	27.2*
6 H	-	2.0	24.6±1.8	30.9
	0.1	2.0	29.8±2.0*	37.7*
I	-	2.0	13.3±1.1	24.9
	0.1	2.0	16.6±1.1*	31.8*
7 B	-	2.0	13.9±1.5	
	0.1	2.0	15.1±1.3	
C	-	2.0	14.8±1.2	
	0.1	2.0	19.2±1.6*	
A	-	2.0	16.8±1.3	
	0.1	2.0	21.5±1.7*	

[a]Adapting dose was given 6 hours prior to challenge exposure and apoptosis was measured after 24 hours using the FADU[b] or TdT[c] assays

[b]Apoptosis induced by the adapting exposure alone has been subtracted from the level in the combined treatments

[c]Percent apoptotic cells as measured by the TdT assay; untreated control levels have been subtracted

exposure was found to have no significant effect on the level of apoptosis induced by the challenge exposure whether the adapting exposure was given 6 hours or 24 hours prior to the challenge exposure.

Table II. The Effect of Various Pre-Exposure Times on Subsequent Radiation-Induced Apoptosis[a]

Time Between Doses (h)	Donor 1	Donor 2	Donor 3	Donor 4
0	10.8 ± 1.1	17.9 ± 1.4	13.9 ± 1.5	14.8 ± 1.2
6	14.0 ± 1.3 *	21.7 ± 1.7 *	15.1 ± 1.3	19.2 ± 1.6 *
24	14.4 ± 1.4 *	23.3 ± 2.0 **	14.7 ± 1.4	18.1 ± 1.4 *

[a] Apoptosis was measured by the FADU assay 24 hours after exposure to 2Gy in cells pre-exposed to a 0.1Gy dose of radiation at the indicated times prior to the challenge exposure. Apoptosis induced by the adapting dose alone has been subtracted from the reported values.
** $p<0.01$, * $p<0.05$

The effect of different adapting doses on the extent of sensitization is shown in Table III. The level of apoptosis induced in cells by a 2Gy challenge dose of radiation was compared to that induced in cells also exposed 6 hours earlier to an adapting dose of 5, 10, or 25cGy. The table shows that in cells from 3 of the 4 donors (donors 1,3, and 4) the level of apoptosis induced by the radiation challenge exposure was significantly increased in cells receiving a prior adapting exposure of 5, 10, or 25cGy. On average, the magnitude of the increase was found to be similar in cells exposed to the 5cGy (27.7 ± 5.4%) or

10cGy (30.4 ± 6.6%) adapting doses. In cells from all 3 donors the magnitude of the increase observed in cells pre-exposed to a 25cGy adapting dose was found to be smaller than that for the 5 or 10cGy doses averaging 19.3 ± 5.3%. However, this difference was not statistically significant. Table III also shows that donor 2 failed to show a significant increase following exposure to any of the 3 adapting doses.

Table III. The Effect of Different Adapting Exposure Doses on Subsequent Radiation-Induced Apoptosis in Human Lymphocytes[a]

Adapting Dose (Gy)	Challenge Dose (Gy)	Donor 1	Donor 2	Donor 3	Donor 4
0.05	-	0.5 ± 0.1	0.2 ± 0.2	0.1 ± 0.2	0.3 ± 0.2
0.10	-	1.0 ± 0.2	0.6 ± 0.3	0.4 ± 0.1	1.2 ± 0.3
0.25	-	2.2 ± 0.2	1.8 ± 0.4	1.1 ± 0.2	3.1 ± 0.9
-	2.0	16.8 ± 1.3	14.4 ± 1.6	9.4 ± 0.9	15.1 ± 1.2
0.05	2.0	20.2 ± 1.5 *	15.3 ± 1.1	12.5 ± 0.7 **	19.6 ± 1.6 **
0.10	2.0	21.5 ± 1.7 *	15.9 ± 1.8	13.1 ± 1.0 **	18.7 ± 1.5 *
0.25	2.0	18.9 ± 1.3 *	15.5 ± 1.4	11.8 ± 0.8 *	18.1 ± 1.4 *

[a] Adapting dose was given 6 hours prior to challenge exposure and apoptosis was measured after 24 hours using the FADU assay. Apoptosis induced by the adapting doses alone have been subtracted from the values reported for the cells receiving the combined treatment

** $p < 0.01$, * $p < 0.05$

Crossadaption: t-Butyl Hydroperoxide versus Radiation

Apoptosis can be induced in resting lymphocytes by either DNA damaging agents or membrane oxidizing agents (Chapter 1, Cregan *et al.* 1999). I tested the possibility that pre-exposure of lymphocytes to a low concentration of t-butyl hydroperoxide (a membrane oxidizing agent) could modify the extent of apoptosis induced by radiation (a DNA damaging agent). The level of radiation-

induced apoptosis was compared in control cells and cells pre-exposed to either 20μM t-butyl hydroperoxide (30 minutes at 37°C) or 0.1Gy of γ-radiation. Table IV shows that in cells from four of the six donors tested, pre-exposure to t-butyl hydroperoxide resulted in a significant increase in sensitivity to apoptosis induced by 2Gy of radiation. The cells from these same four donors also showed a significant increase in sensitivity in response to the radiation adapting dose. The mean relative increase in radiation-induced apoptosis was 27.8 ± 3.5% in lymphocytes pre-exposed to the radiation adapting dose and 28.9 ± 6.3% in cells pre-exposed to t-butyl hydroperoxide. Furthermore, the table also shows that t-butyl hydroperoxide was effective at causing sensitization to radiation-induced apoptosis when the cells were exposed to the drug at either 0°C or 37°C.

I also tested the ability of t-butyl hydroperoxide or radiation adapting exposures to modify the extent of apoptosis induced by a subsequent t-butyl hydroperoxide challenge exposure (200μM, 30 min at 37°C). Table IV shows that in the same donor cells (experiments 3, 4, and 6) in which pre-exposure to radiation or t-butyl hydroperoxide resulted in a significant enhancement in radiation-induced apoptosis, neither pretreatment significantly affected the level of apoptosis induced by the t-butyl hydroperoxide challenge exposure, nor was there any effect in the cells (donor 5) that did not respond to a radiation pretreatment with respect to radiation-induced apoptosis.

Table IV. The Effect of Adaptive Treatment with Low Dose Radiation or Low Concentrations of t-Butyl Hydroperoxide on the Induction of Apoptosis by a Subsequent Challenge Exposure of Radiation or t-Butyl Hydroperoxide[a]

Adaptive Treatment	Challenge Treatment	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6
γ-rays	-	1.1 ± 0.4	0.4 ± 0.2	0.7 ± 0.2	0.9 ± 0.2	1.3 ± 0.4	0.3 ± 0.2
t-BuOOH (37°C)	-	0.8 ± 0.3	0.3 ± 0.2	0.4 ± 0.2	1.2 ± 0.4	0.7 ± 0.2	1.1 ± 0.3
t-BuOOH (0°C)	-		0.1 ± 0.2				
-	γ-rays	19.6 ± 1.7	10.3 ± 1.2	12.3 ±1.1	19.6 ± 2.1	15.2 ± 1.9	13.6 ± 1.2
γ-rays	γ-rays	20.8 ± 1.8	13.7 ± 1.4 *	15.4 ± 1.3 *	24.3 ± 2.0 *	16.9 ± 1.7	17.5 ± 1.4 *
t-BuOOH (37°C)	γ-rays	18.6 ± 1.8	14.2 ± 1.4 *	15.1 ± 1.1 *	25.7 ± 2.1 *	16.1 ± 1.6	16.7 ± 1.4 *
t-BuOOH (0°C)	γ-rays		13.0 ± 1.1 *				
-	t-BuOOH			19.4 ± 2.1	36.6 ± 3.5	23.7 ± 2.2	31.1 ± 2.8
γ-rays	t-BuOOH			20.2 ± 3.4	38.1 ± 3.5	24.2 ± 2.0	30.5 ± 3.1
t-BuOOH (37°C)	t-BuOOH			18.3 ± 2.2	34.4 ± 3.4	22.4 ± 2.3	28.8 ± 3.0

[a] Apoptosis was measured by the FADU assay 24 hours after exposure to either 2Gy of γ-rays or 200μM t-BuOOH (at 37°C) in control cells or cells exposed 6 hours earlier to either 0.1Gy γ-rays or 20μM t-BuOOH. Apoptosis induced by the adapting exposures alone have been subtracted from the corresponding combined treatment values.

* $p<0.05$

DISCUSSION

Characterization of Radiation-Induced Sensitization to Radiation-induced Apoptosis

An adaptive response has previously been described in lymphocytes showing that exposure to a low dose of radiation can induce a chromosomal repair mechanism such that cells, including mitogen stimulated lymphocytes, subsequently exposed to a high dose display a reduced frequency of chromosomal aberrations as compared to non-adapted cells (Olivieri *et al.* 1984, Wiencke *et al.* 1996). The results presented here demonstrate that lymphocytes exposed to a low dose of radiation can become sensitized to the induction of apoptosis by a subsequent radiation exposure. The induction of this sensitizing response was also shown to exhibit both inter- and intra-individual variability, similar to that seen when induced resistance to chromosomal aberrations was measured (Bosi and Olivieri 1989). The lack of a significant response in cells from certain donors did not appear to result from different adapting exposure requirements as the response could not be elicited by changing the adapting exposure dose (Table III) or by altering the time between the two radiation exposures (Table II). The indication that cells from one donor responded differently on different occasions (Table I) suggests that the induction of this response may be dependent on the physiological status of the lymphocytes at the time of isolation. Exogenous factors such as exercise habits, alcohol consumption, diet, psychological stress, or illness may contribute to this observed variability.

The degree of sensitization induced by the low dose radiation exposure was found to be significantly greater when the adapting exposure was given 6 hours (or 24 hours) prior to the challenge exposure than when it was given immediately before (Table II). This result suggests that the sensitization effect of the low dose radiation exposure involves an induced response and does not simply result from an additivity of the lesions produced by the two radiation exposures. Furthermore, the results demonstrate that the induced signal is fully developed by 6 hours and persists for at least 24 hours.

I have previously reported that apoptotic cell death could be induced in human lymphocytes by a kinetically slow process following exposure to DNA damaging agents like ionizing radiation or a kinetically fast process following exposure to membrane oxidizing agents such as t-butyl hydroperoxide (Chapter 1, Cregan *et al.* 1999). These agents were shown to trigger the apoptotic process by distinct mechanisms consistent with activation by DNA damage and membrane oxidative processes respectively. Furthermore, it was shown that apoptotic cell death was not induced in cells exposed to t-butyl hydroperoxide at 0°C and subsequently incubated at 37°C conditions which produced rapidly repaired single strand breaks. In the present study I have shown that increased sensitivity to radiation-induced apoptosis could be triggered in cells pre-exposed to either a low dose of radiation or a low concentration of t-butyl hydroperoxide (Table IV). On the other hand, these pretreatments did not significantly modify the level of apoptosis induced by a challenge exposure of t-butyl hydroperoxide. Low concentrations of t-butyl hydroperoxide triggered sensitization to radiation-

induced apoptosis following drug exposures at 0°C or 37°C. Since I have previously shown that (high concentrations of) t-butyl hydroperoxide could not trigger apoptosis following drug exposures at 0°C, this result suggests that the signalling mechanism involved in the sensitizing response (to radiation-induced apoptosis) is different than that which signals the apoptotic process itself. In addition to its membrane oxidizing capabilities (Masaki *et al.* 1989), t-butyl hydroperoxide is also known to be effective at producing DNA single-strand breaks at either 37°C or 0°C (Latour *et al.* 1995, Cregan *et al.* 1999a). Therefore, the ability of exposure to t-butyl hydroperoxide at 0°C to trigger the sensitizing response but not apoptosis suggests that rapidly repaired single-strand breaks may be effective lesions for the induction of the sensitizing process, but not the apoptotic process itself (Chapter 1, Cregan *et al.* 1999a). It is also possible, however, that this sensitization could be triggered by a signal not associated with DNA damage. On the other hand, neither DNA damaging events nor membrane oxidizing events resulted in any detectable sensitization to the kinetically fast apoptotic process induced by a membrane oxidizing agent

Adaptive Response Pathways

An adaptive response has previously been described in which it has been proposed that exposure to low dose radiation can induce a chromosomal repair mechanism (Wiencke *et al.* 1986, Wollf *et al.* 1989). In the present study we have shown that unstimulated lymphocytes pre-exposed to a low dose of radiation become sensitized to the induction of apoptosis by a subsequent

radiation exposure. It is possible that both of these pathways are stimulated by the adapting dose of radiation but that the relative activity of these pathways may be dependent on the cell type and /or the growth state of the cell. Thus in fibroblasts or mitogen stimulated lymphocytes which are known to be relatively resistant to radiation-induced apoptosis (Lowe *et al.* 1993a, Harris and Lowenthal 1985, Sellins and Cohen 1987) the adapting exposure appears to preferentially activate the chromosomal repair mechanism (Azzam *et al.* 1992, Shadley and Wolff 1987), increasing the rate at which chromosomal breaks are repaired (Azzam *et al.* 1994) and the fidelity of that repair (Sanderson and Morley 1986, Kelsey *et al.* 1991, Azzam *et al.* 1994). On the other hand, in apoptotically sensitive cell types such as unstimulated lymphocytes, the adapting exposure appears to preferentially induce sensitization to radiation-induced apoptosis.

The activation of p53 in response to DNA damage is known to induce distinct cellular processes depending on the cell type and growth status of the cell (reviewed in Ko and Prives 1996). In certain cell types, including resting lymphocytes, the induction of p53 in response to DNA damage is known to be involved in the activation of apoptotic cell death (Clarke *et al.* 1993, Lowe *et al.* 1993b, Strasser et al 1994). In other cell types including fibroblasts and cycling lymphoid cells, the DNA damage induced p53 response involves activation of a G_1 cell cycle arrest mechanism (Kastan *et al.* 1992). It has been proposed that cells activate cell cycle arrest (cell cycle checkpoints) in response to DNA damage to allow DNA repair to occur before cells reenter the cell cycle, thereby

preventing fixation of the damage during mitosis (Hartwell and Kastan 1994). Therefore, the induction of a cell cycle delay mechanism would be expected to reduce the frequency of chromosomal aberrations in the irradiated population. In fact, recent studies on the adaptive response have shown that cell cycle delay is extended in radiation adapted cells (Meyers *et al.* 1995, Mitchel 1995). Different groups have reported increased levels of a variety of mRNAs in cells exposed to low doses of ionizing radiation. The mRNAs identified to date appear to code for proteins known to be involved in cell cycle control, including p53 (Meyers *et al.* 1992; 1995, Woloschak and Chang-Liu 1990). Thus, in cell systems in which p53 has been implicated in the G_1 cell cycle arrest/repair mechanism, the adaptive response to radiation appears to involve an induced repair capacity (Azzam *et al.* 1992, Shadley and Wolff 1987, Seong *et al.* 1995). On the other hand, in cell systems in which the p53 response leads to the induction of apoptosis, such as the unstimulated lymphocytes studied here, the adaptive response to radiation appears to involve sensitization to apoptotic cell death. This suggests a possible central role of p53 in the adaptive response to radiation-induced apoptosis. Low doses of ionizing radiation may alter the induction of p53 which could result in modification of the cell cycle arrest or apoptotic cell death pathways depending on the cellular system.

In some studies on adaptive response, an increase in survival was observed in adapted cells as measured by a clonogenic endpoint (Azzam *et al.* 1992, Shadley and Dai 1992). These studies, however, were done with proliferating cells in which cell death following irradiation is a slow process

dependent on mitotic activity. This mode of death has been termed reproductive or clonogenic cell death because survival is assessed by the ability of cells to continue to divide and form a colony. The correlation between the extent of reproductive cell death and chromosomal aberrations suggests that misrepaired DNA lesions are ultimately responsible for the loss of clonogenic survival (Joshi *et al.* 1982, Obe *et al.* 1992). Thus, the increased survival observed in adapted cells would be consistent with the induced repair capacity in these cells.

In other studies on the adaptive response it was shown that exposure to low dose radiation could reduce the frequency of mutations in G_0 lymphocytes subsequently exposed to a mitogen (Sanderson and Morley 1986) or neoplastic transformation in C3H 10T1/2 cells (Azzam *et al.* 1994). However, this was not associated with a survival advantage. The authors suggested that an error-free DNA repair process was induced and speculated that "the types of lesions leading to mutation and lethality are different and that differential repair of pre-mutational and potentially lethal lesions is possible". Interestingly, the cell systems used in these studies are known to be sensitive to radiation-induced apoptosis (Delic *et al.* 1993, Tomei *et al.* 1988). Therefore, it is possible that the reduced frequency of mutations observed in these studies could result from an enhanced removal of genetically damaged cells by an apoptotic mechanism.

Possible Mechanisms of Radiation-Induced Sensitization to Radiation-induced Apoptosis

It has been suggested that the sensitivity of cells to apoptotic stimuli is determined by the relative expression of pro-apoptotic and anti-apoptotic genes (Oltvai and Korsmeyer 1994). It is possible that low dose radiation could alter the relative expression of these genes, thereby altering the cells susceptibility to subsequent apoptotic stimuli. It has been shown in a number of different cell systems that overexpression of bcl-2 can protect against the induction of apoptosis by ionizing radiation and other genotoxic agents (Sentmen *et al.* 1991, Miyashita and Reed 1992, Zhong *et al.* 1993, Strasser *et al.* 1994, Korsmeyer 1995). Overexpression of bax, on the other hand, has been shown to promote apoptosis (Zhan *et al.* 1994, Sakukara *et al.* 1996). Unlike p53 though, overexpression of bax itself does not trigger apoptosis, but requires a cell death signal (Oltvai *et al.* 1993). Bax can form homodimers or it can heterodimerize with bcl-2 (Oltvai *et al.* 1993), and it is thought that the ratio of bcl-2 to bax predetermines the cell's susceptibility to a given apoptotic stimuli (Chresta *et al.* 1996, Thomas *et al.* 1996). Thus, apoptosis is triggered if the level of apoptotic signals reaches some critical threshold level which is determined by the relative expression of bcl-2 and bax, ie. the balance of bax homodimers which favour cell death to bcl-2/bax heterodimers that inhibit death. The tumour suppressor p53 has been shown to alter the relative balance of this system by increasing the expression of bax (Miyashita *et al.* 1994, Miyashita and Reed 1995) and inhibiting the expression of bcl-2 (Halder *et al.* 1994). Since p53 is known to be

induced by radiation-induced DNA damage (Kastan *et al.* 1991, Fritsche *et al.* 1993, Nelson and Kastan 1994) it is possible that low dose radiation could alter the relative expression of bcl-2 and bax proteins thereby lowering the apoptotic signal threshold requirements. Therefore, upon a subsequent radiation exposure a greater number of cells might be expected to reach this lower threshold level.

In summary we have shown that exposure to a low dose of radiation or an agent which produces DNA single strand breaks, can increase the sensitivity of lymphocytes to subsequent radiation-induced apoptosis but not to apoptosis induced by membrane oxidizng agents. We suggest that this increased sensitivity may represent an adaptive response mechanism which could reduce the probability that genetically damaged cells will proliferate, thereby lowering the cancer risk associated with exposure to ionizing radiation or DNA damaging agents.

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SUMMARY

The results presented here demonstrate that apoptosis can be induced in human lymphocytes by two distinct pathways. Lipophilic oxidizing agents trigger apoptosis through a rapid induction pathway that does not require active protein synthesis and which is initiated by membrane oxidative processes. Ionizing radiation and the other DNA damaging agents, on the other hand, trigger apoptosis by a slow induction pathway which requires active protein synthesis and that is initiated by a persistent signal related to slowly repaired or unreparable DNA damage.

Furthermore, we have demonstrated that exposure to a low dose of radiation or an agent which produces DNA single strand breaks, can increase the sensitivity of lymphocytes to subsequent radiation-induced apoptosis, but not to apoptosis induced by membrane oxidizng agents. We suggest that this increased sensitivity may represent an adaptive response mechanism which could reduce the probability that genetically damaged cells will proliferate, thereby lowering the cancer risk associated with exposure to ionizing radiation or DNA damaging agents.