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RADIATION INDUCED APOPTOSIS IN HUMAN MALIGNANT GLIOMA CELLS

BY

JACQUELINE M. LEITHOFF

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Radiation Induced Apoptosis in Human Malignant Glioma Cells** submitted by **Jacqueline M. Leithoff** in partial fulfillment of the requirements for the degree of **Master of Science.**

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ABSTRACT

Apoptosis, or physiological cell death may be a very important factor in tumor growth kinetics. This process can be induced in both normal and tumor cells with ionizing radiation and chemotherapeutic drugs. Our studies were designed to characterize apoptosis following radiation treatment and photodynamic therapy. Two radiosensitive (M059J and M071) and two radioresistant (M059K and M016) cell lines were used. Apoptosis was measured using agarose gel electrophoresis, zero integrated gel electrophoresis, propidium iodide staining and flow cytometry. In addition, apoptosis was studied in EMT6 mouse mammary carcinoma cells after photodynamic therapy. Four experimental objectives included the following: 1) Which method or combination of methods may be most useful for measuring apoptosis in human tumor cells both qualitatively and quantitatively? 2) Does inter- and intratumoral heterogeneity exist with respect to apoptotic suseptibility? 3) Could the molecular pathways involved in apoptosis and radiation sensitivity be similar? 4) Is apoptosis an important mode of cell death in photodynamic therapy? The glioma cell lines tested varied in their suseptibility to apoptosis, with the DNA dsb repair deficient line, M059J, being most sensitive. Unlike cells of lymphoid origin, no glioma cell lines showed the classic pattern of DNA "ladders" following radiation treatment. Rather, ZIFE results indicate the process of endonucleolysis was halted at the 300 kb fragment size. Chromatin condensation and a post-irradiation G1 shift were also observed in apoptotic cells. However, only in M059J cells did apoptotic death contribute significantly to radiation sensitivity. Studies with EMT6 cells treated with photodynamic therapy showed a similar pattern of endonucleolysis.

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I. INTRODUCTION

1.1 APOPTOSIS: PHYSIOLOGICAL CELL DEATH

Throughout evolution cells have adapted to adverse environmental conditions by a wide range of responses. An intricate balance of intercellular connections and intracellular signalling mechanisms provide cells with homeostatic monitoring ability that allows for advanced forms of life. Just as an individual cell must balance anabolic and catabolic reactions, a multicellular organism must equalize the rate of cell generation and death in order to maintain a constant size and function. Consequently organisms have evolved mechanisms to remove individual cells which are no longer needed or which function abnormally.

Cancers arise when changes in these cell mechanisms cause clonal expansion and accumulation dependent on the rates of cell division vs cell death. Cell death then, could be considered to be a specific physiological process that is regulated and orchestrated by the cell rather than the effect of secondary factors in the environment. This "physiological cell death" has been known for decades but was first formalized by Kerr, Wyllie and Currie[1] in 1972 who described the ultrastructural changes that are characteristic of these dying cells. They coined the word *apoptosis* to describe this process. Apoptosis is derived from a Greek word used to describe the dropping of leaves from trees.

This process is also referred to as *programmed cell death* because it appears to be a programmed event in embyogenesis, tissue regression and atrophy, and the normal cell turnover in hematopoetic systems. In evolutionary terms, this process would more realistically be called *programmed cell survival*[2] for the purpose of such a system is probably maintenance of genetic fidelity,

minimization of phenotype variation and elimination of genetic alteration. This mechanism can not only account for aberrant cell deletion but the deleted cells may be indirectly responsible for the survival of the other normal cells.

If apoptosis is indeed a protective mechanism for maintaining genetic fidelity it would require endogenous cellular factors capable of regulation. As with all aspects of physiology there is a compelling association with molecular events in the cell. A continuous stream of signals from outside the cell to its interior and exchange of signals between the nucleus and the cytoplasm are extremely important in order for the cell to be able to manipulate its genomic and metabolic processes according to external conditions. With an understanding of this *signalling net* it may be possible to understand the regulation of apoptosis in order to manipulate the process for therapeutic benefit.

1.2 APOPTOSIS AND CARCINOGENESIS

Cancer can be considered to be a disturbance in the growth control mechanism of the cell. However, a cell's ability to use apoptosis as a regulatory mechanism may be even more important than its proliferative ability. Some cancer cells divide more slowly than do some normal cells, nonetheless, the neoplasm still expands because of a prolonged life span in the cancer cells.

A cell can respond to injury (for example, radiation or chemotherapeutic drugs) in several ways: 1) it can delay cell division until the damage is repaired, 2) it can undergo apoptosis or 3) it can progress without interuption through the cell growth cycle. It has been suggested that cells that have become malignantly transformed have aquired the ability to bypass the first two

checkpoints and move quickly through the proliferative cycle unimpeded. For example, certain types of oncogenic viruses (the E1A adenovirus) integrate into host genes that are associated with apoptosis regulation, thereby effectively blocking apoptosis[3].

Many tumor cells, however, retain the ability to die by apoptosis. It has been suggested that apoptosis may be the most significant component of the well established continuous cell loss seen in tumors[1] but quantification of this loss has proven to be very difficult and documented cases are very few.

Abnormal apoptotic control can promote cancer development by allowing the accumulation of the dividing cells as well as any genetic variants. What are the factors that are important for the cell to *decide* which path it will choose? Many of the genes that have been found to be associated with apoptosis are also linked to the regulation of proliferation. Studies with cells that have been transfected with the *myc* oncogene[4] suggest that a cell can receive mixed signals as to whether to divide or die. In biochemical terms such *mixed signals* may consist, for example, of both a signal to stop cycling (due to deprivation of an essential amino acid) and a signal to proliferate (due to constitutional up-regulation of *c-myc).* In such instances apoptosis is triggered.

Apoptosis can also function as a brake on cell proliferation. For a proliferating cell to divide at least two external stimulatory signals are required - a *competence* signal and a *progression signal.* The competence signal induces metabolic events that are common to both replication and apoptosis. The second progression signal will commit the cell to replication. Apoptosis can be considered a *default pathway* that destroys those cells that are unable to cross a checkpoint before DNA replication[5]. This occurs in competence activated cells

when a progression signal is absent or abnormal or when a cell does not repair damage within a critical period of time.

A better understanding of the intracellular signalling pathways that control the balance between cell proliferation and apoptosis may lead to the development of novel anti-cancer therapies.

1.3 THE 'CLASSIC MODEL' OF APOPTOSIS

Much of the evidence for the existence and regulation of apoptosis has come from the early studies[1,6] in rodent thymocytes or T cell hybridomas. In using these models, researchers sought to identify characteristics that would allow them to discriminate between apoptotic cell death, which is considered to be initiated within the cell, and necrotic cell death which is initiated by factors in the cell environment . The *classic model* described by Wyllie[15] is used as the characteristic apoptotic phenotype. This model includes the following:

- apoptosis affects scattered individual cells rather than cells in a particular area.
- the process can be provoked by various pathological stimuli but once initiated, procedes quickly.
- cell fragmentation occurs without leakage of the cellular contents.
- removal of the cells does not cause an inflammatory response. **This is the crucial feature - cell death occurs without damage to the adjacent cells.**

However, it is now known that the apoptotic process can vary dramatically not only among cell types but also according to the induction agent used. For example, *lethal agents* have been categorized as such according to their ability

to induce cell death by apoptosis. X-ray and γ ray radiation[7,8], treatment with the Ca^{+2} ionophore A23187[9,10], cytotoxic drugs or nucleosides,[11] growth factor withdrawal[12], antibody treatment [13] or RNA /protein inhibition [14] have all been shown to alter the rate of normal apoptotic response in some, but not all cells. Some examples of these alterations will be discussed in later sections.

1.3.1 Morphological Changes

Distinct morphological changes have been extensively documented in many cell types. Time lapse cinematography of cultured fibroblasts has shown that these changes occur rapidly. The first change noted is that the plasma membane appears to boil before the cell explodes into apoptotic fragments[4]. Photomicroscopy has shown cell shrinkage and distortion with the cell membrane becoming convoluted. The shrinkage of the cell is due to a loss of cytoplasmic volume and the condensation of cytoplasmic proteins, with most of the cytoplasmic organelles remaining intact. The induction of a transglutaminase[16,17], an enzyme that crosslinks proteins, appears to be responsible for this distortion.

In the next stage, membrane rufflings and blebbings lead to cellular fragmentation. There is a dramatic increase in buoyant density and the cells lift off the surface on which they normally grow. It has been proposed[18] that in addition to net movement of fluid out of the dying cells (up to one half of the cell's volume within a few minutes) there is also inhibition of the sodium/ potassium/chloride co-transporter system, leading to ionic imbalance in the cell.

In the last stage of apoptosis, the neighboring cells (*in vitro*) or

macrophages (*in vivo*) phagocytize the apoptotic fragments and completely degrade them. This seems to be directed by a new molecular structure that is being revealed on the surface of the apoptotic cell[19,20]. The signal may be a glycan that is recognized by the macrophages which then engulf the cell fragments without eliciting the inflammatory response normally found with necrotic cell death.

1.3.2 Biochemical Changes

Perhaps the most important changes in the apoptotic cell occur within the nucleus where the chromatin can be seen to condense into dense granular fragments and aggregate under the nuclear membrane. Further biochemical characterization of these fragments[9,21] has shown that the chromatin is cleaved specifically between the nucleosomes reducing the DNA to integer sized fragments. This is due to the activation of an endogenous endonuclease and results in what is commonly referred to as the *DNA ladder*. Classically, this characteristic alone has been considered criteria for the existence of apoptosis. Using agarose gel electrophoresis the DNA ladder characteristic will appear in 180 - 200 base pairs (bp) multiples running in discrete bands, reflecting the controlled and regulated manner in which the DNA is cleaved. By comparison, DNA from cells dying of necrosis, a process in which the contents of the cell are indiscriminately fragmented, produce a *smear* on the agarose gel.

1.3.3 Cells of Hematopoietic Lineage

It has been suggested recently that although this model may serve as a

generalized description of cellular apoptosis, it should be recognized that cells of the blood and lymph systems may be unique in their apoptotic machinery. These tissues exquisitely regulate the life and death of their constituent cells. Thymocytes are delicately poised on the brink - positive selection resulting in maturation into functional CD^{+4} or CD^{+8} cells - or negative selection resulting in death by induction of apoptosis[22]. It is also known that a single receptor ligand interaction can lead to strikingly different cellular responses[23,24]. For example, binding of tumor necrosis factor (TNF) to its receptor in immature CD^{+4} cells will signal apoptosis. In contrast, binding of TNF to its receptor in mature post-thymic T cells will intiate entry into S phase.

This complexity displayed within one type of tissue only begins to mirror the complexity found among tissues of different types. An additional complexity is the fact that many apoptosis initiating agents are known, and each may initiate apoptosis at a different point in the multimechanistic pathway model. One thing remains certain however, the ultimate event is the careful dismantling of the cell to *apoptotic bodies,* the membrane bound cell fragments that were first described by Wyllie and Kerr many years ago.

1.4 APOTOSIS ASSAYS: NEW METHODS FOR DETERMINATION OF MORPHOLOGIC AND BIOCHEMICAL CHARACTERISTICS.

The fundamental determination of apoptosis has classically involved agarose gel electrophoresis of the DNA in order to detect DNA ladders. Further studies with a variety of normal and cancer cells have suggested that not all cells have the ability to fragment their DNA to such small pieces[25] and as a

result, the DNA ladder criteria may not be absolute. This has led researchers such as Collins *et al.* [26] to suggest the following:

> " The hypothesis that apoptosis is a discrete phenomenon is based at present, on a concordance of distinctive ultrastructural features, DNA degradation pattern and circumstances of occurrence. This concordance is clearly not absolute. In experiments designed to determine whether apoptosis can be clearly identified in molecular terms, it is important that the cell death taking place be described as comprehensibly as possible. We suggest that, at very least, both ultrastructural features and DNA degradation patterns be recorded. The inclusion of quantitative and termporal data to help to define its kinetics would also be of considerable value." (Collins *et al.* p 453)

The identification of apoptotic cells has become complex. There is growing evidence that in many cell systems apoptosis may be *atypical,* lacking one or more of the features that characterize classic apoptosis in thymocytes. In other cases, some of the features associated with apoptosis, such as internucleosomal DNA degradation may also accompany necrosis. The use of the conventional agarose gel electrophoresis method would fail to identify any atyptical apoptotic cells. Even when the apoptosis is deemed typical none of the following methods when used alone can provide total assurance of detection. The applicability of each of the methods varies depending on the cell system, the nature of the agent used to induce apoptosis, the particular information being sought and the technical restrictions.

1.4.1 Fluorescence and Light Microscopy

The most conspicuous cellular changes that signal the presence of apoptosis can be seen with the light microscope. Nuclear condensation and

formation of granules under the nuclear membrane can be seen. With the use of vital dyes it is possible to estimate how long the cells remain alive. It has been found that during the phase of condensation a cell can still exclude vital dyes and so must retain some normal enzyme function[27]. At this time cells also display an abrupt increase in buoyant density. However, cells undergoing apoptosis show very low rates of protein and RNA synthesis and after only a few hours begin to lose the capacity for volume homeostasis.

If the chromatin of cells is stained by a fluorescent dye such as acridine orange or propidium iodide, changes can be observed by fluorescence microscopy[28]. The condensed chromatin becomes a uniform compaction of beadlike structures of different sizes. If the cells are followed for various times after the induction of apoptosis, it is possible to show with fluorescence microscopy the fragmentation of the cell and formation of apototic bodies.

Another interesting assay that involves the fluorescent and light microscope is *in situ* end labelling (ISEL). This method is based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to the 3' OH ends of DNA[29]. The enzyme is used to incorporate biotinylated (or fluorescein conjugated) deoxyuridine at sites where the DNA has been broken. The signal can then be amplified with avidin peroxidase (for light microscopy) or fluorecein (for fluorescent microscopy) enabling the identification of the apototic cells with a positive signal. This method is specifically used to quantify apoptosis in cell populations and tissues without disturbing the natural postioning of the cells. Some authors[30] have suggested that this method provides further clues with respect to the fine details of the process in tissues, for example - apoptotic cells appear in clusters and the period through which dying cells can be revealed is

relatively short - perhaps just a few hours in some tissues.

1.4.2 Electron Microscopy

In addition to identifying the nuclear condensation, compaction of organelles and appearance of protuberances on the cell surface of apoptotic cells, electron microscopy is able to show other distinct differences between apoptotic and necrotic cell death[26]. Necrotic cells show swollen cellular components and disrupted membranes, with chromatin distributed in small ill defined masses. Apoptotic cells lose contact with their neighbors and specialized areas of the cell membranes such as junctional complexes and microvilli disappear. On scanning electron microscopy, dilated cisternae (considered to be endoplasmic reticulum and golgi) fuse with tne cell surface giving the bizarre *cratered* appearance .

1.4.3 Flow Cytometry

Flow cytometry allows one to rapidly and accurately measure individual cells in large cell populations. As the term implies flow cytometry is a process in which measurements are made while the cells pass single file through a measuring apparatus in a fluid stream. DNA cleavage and the preservation of at least some function of the cell membrane provide the basis for the development of most of the flow cytometric assays to specifically identify apoptotic cells. Measurement of cellular DNA content allows cells to be identified on an DNA histogram or bivariate DNA/protein contour map. As the cells with fractional DNA content represent an apoptotic population, it is possible to quantify the proportion of apoptotic cells in a given population. The

disadvantage of this method is that under certain conditions, apoptotic cells may be indistinguishable from normal cells. This could occur when DNA degradation in a given apoptotic cell is not much advanced and as a result most of the DNA in that cell will be still of high molecular weight. This causes an overlap between the normal and apoptotic cell populations which leads to underestimation of the latter.

The fixation method of cells is inadequate to preserve the degraded low molecular weight DNA inside the apoptotic cells (due to the fragmenting by the characteristic endonuclease). This portion of DNA *leaks out* during subsequent rinsing and staining procedures and therefore less DNA in these cells is stained with the DNA fluorochrome. The appearance of cells with low DNA stainability (sub G_1 content) can be considered to be a marker of cell death by apoptosis[31].

Light scatter also changes during apoptosis due to morphological and nuclear density changes. Reduced ability to scatter light in the forward direction and either an increase or no change in the 90° light scatter characterize cells in the early phases of apoptosis. In later stages both the forward and right angle light scatter signals are decreased [32].

Fluorescent TdT labelling can also be used in conjunction with flow cytometry. This method can allow for simultaneous detection of DNA strand breaks with analysis of DNA content. This may be most useful for identifying the cell cycle position of cells in both the apoptotic and non apoptotic populations[33].

1.4.4 Agarose Gel Electrophoresis

Conventional agarose gel electrophoresis has classically been used to identify DNA cleavage at the linker regions between nucleosomes, resulting in the DNA ladder of apoptosis. But recently the relationship of condensed chromatin and the detection of this endonucleolytic cleavage has been questioned. Changes in the integrity of the DNA may be more optimally evaluated with pulsed field gel electrophoresis (PFGE) because this technique allows analysis of DNA with molecular weights of up to 2 mbp whereas the conventional gel electrophoresis is restricted to the analysis of fragments 20 kbp and less. PFGE separates large DNA fragments by periodic changing of the orientation of the electric field, forward and backward with respect to the DNA. Zero integrated field electrophoresis (ZIFE) involves a modification of the pulse field model in which lower voltage gradients in the reverse field than the forward field and longer times in the reverse direction than in the forward direction are employed. Maximum resolution occurs under these conditions with sharp bands and minimum band inversion. The ratio of the forward and reverse voltage X duration is close to 1, hence the name zero integrated field electrophoresis.

Examination of higher order DNA cleavage using PFGE has shown discrete fragments of approximately 300 and/or 50 kbp in several cell types prior to, concomitantly with, or in the absence of the classic internucleosomal cleavage[34]. The size of these larger DNA fragments imply that the rosettes (300 kb) or loops (50 kb) of DNA are somehow released from the nuclear scaffold in an orderly manner [35].

1.4.5 Which assay to choose ?

> "Regardless of the assay used to identify apoptosis, the mode of cell death should be positively identified by inspection of cells under light or electron microscopy. Morphological changes during apoptosis have a very specific pattern and should be deciding in situations when there is ambiguity regarding the mechanism of death" (Darzynkiewicz *et al.* [27] p 19).

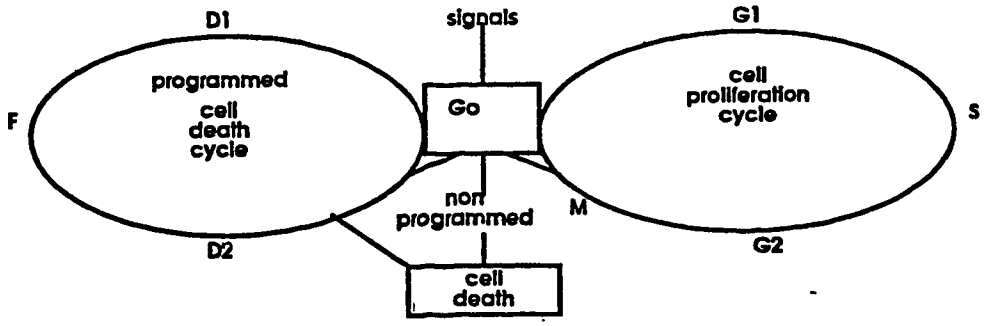
1.5 THE MOLECULAR MECHANISMS OF APOPTOSIS

Much of the current enthusiasm for understanding the mechanisms of apoptosis is founded on an attempt to understand the clinical problem of cancer. The cancer cells ability to undergo apoptosis spontaneously is associated with the progression of tumor malignancy. This has been correlated with the expression of several oncogenes or tumor suppressor genes such as *bcl-2, c-myc, ras* or *p53* and may be prognostic of treatment response [36]. Research is underway in many laboratories to find the means to modify the apoptotic response of normal and tumor cells to various agents. It is expected that a knowledge of the molecular mechanisms of apoptosis will be helpful in developing new antitumor strategies.

Although our understanding of the pathways that induce apoptosis is incomplete, we do know that some sort of *cellular trigger* initiates a signal which is then modulated and transduced through the cell to the nucleus with the ultimate response being the systematic dismantling of the cellular machinery[37] . We also know that the apoptotic process is inextricably linked to the cell cycle control, but the relationship between the two is far from clear and appears to vary among cell types and induction stimuli.

Berges *et al.*[38] have suggested the following model to explain the link between proliferation and apoptosis. Their model requires that the typical cell cycle model which controls cell number be modified. In its place they propose a *redefined cell cycle*, composed of a multicompartment system in which cells have at least three options:

1. to stay in G_0 - not undergoing either proliferation or death.
2. to undergo proliferation (G_0 -> G_1 -> S ->G_2 ->M).
3. to undergo cell death - either programmed (G_0 -> D_1 -> F_1 -> D2-> cell death) or non programmed (necrosis).



D1 is the period in which new gene and protein expression occurs

F is the period in which DNA fragmentation occurs

D2 is the period in which the cell itself fragments to apoptotic bodies

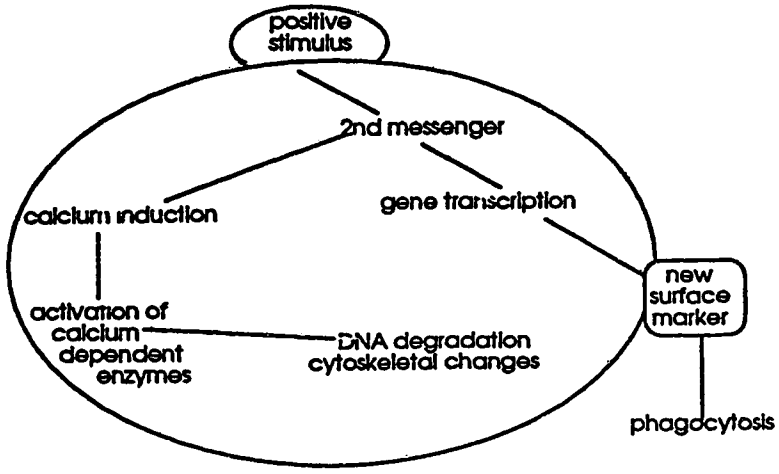
The regulation of proliferation in the cell is important for apoptosis induction. Evans *et al.*[39] found that cisplatin will induce the same number of lesions in the DNA of both quiescent (G_0) cells and proliferating cells, but only the proliferating cells will undergo apoptosis. This has been interpreted as

evidence that some sort of *DNA sensoring of lesions* occurs in cycling cells before they enter G_2/M, and that this lesion recognition is necessary for the induction of apoptosis in these cells when they enter the following G_1 phase. This process may be specific for T lymphocytes, but it is also possible that in other cell types, DNA damage recognition by the sensor could induce apoptosis in any phase of the cell cycle.

Another characteristic of the cell that is important for apoptosis response is the cells' state of differentiation. Generally, as cells in a tissue move toward a differentiated state (for example, cells in hierarchical tissue such as intestinal crypt cells), they lose the ability to die by apoptosis induced by radiation[40]. This could prove most important for cancer cells tend to be genetically unstable and undifferentiated in relation to other more slowly proliferating normal cells. The ability to lose apoptotic suseptibility would give these cells a selective advantage and allow accumulation of the cells as tumors.

1.5.1 The Single Pathway Model.

One difference between the undifferentiated and the differentiated cells is their signalling pathways; as differentiation proceeds, some pathways are closed and new ones are opened. The complexity of this signalling network is still not well understood, but appears to require expression of RNA and protein. Carson *et al.*[41] have proposed the following as a general model for the common metabolic events in apoptosis:



However, many discrepancies have arisen when researchers have tried to manipulate the proposed mechanisms in this model. Some of these complexities and their possible interpretations are discussed below.

1.5.2 Cohen' s Multi Mechanism Model

Cohen *et al.*[42] argue against a universal requirement for de novo expression of proposed *death genes* and offer the following model for the discrepancies seen between different cell types:

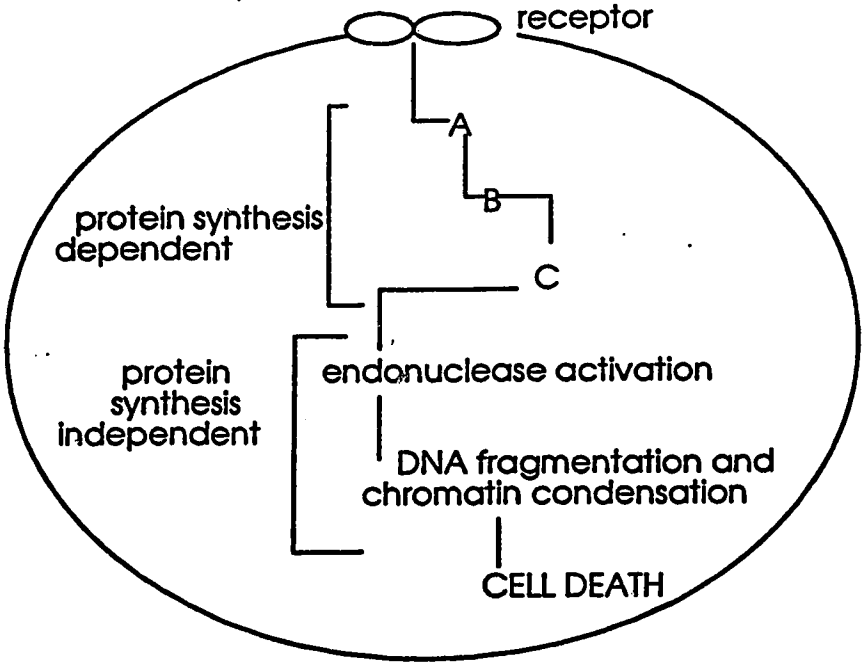
1. Much of the evidence for protein and RNA synthesis requirement comes from rodent thymocytes. These cells may be exceptions to the norm.

2. In some instances, protein and RNA inhibitors have been shown to block apoptosis, however, the possibility that this response was due to the effects of the inhibitors on other cellular processes has not been

excluded.

3. Cyclohexamide inhibition of protein synthesis resulting in apoptosis suggests a requirement for *continuous* macromolecular synthesis. This does not establish that *de novo* protein synthesis is required.

From these observations we can no longer assume that there is a single apoptotic pathway available to the cell. Perhaps as Martin[43] has suggested there may be one protein dependent pathway and a protein independent pathway. Alternatively, there may be one pathway in which only early events require protein synthesis, but that latter steps along this pathway could be accessed at several points.



For example, a certain stimulus (cytotoxic T cell mediation) may interact

with the pathway at a fairly advanced stage. This would bypass the early events that would normally be required for apoptosis induced by other means (chemotherapeutic drugs or ionizing radiation).

This model helps to explain some of the previously found conflicting evidence. For example:

1. Different signals can induce different responses.
2. Induced protein synthesis can differ in time and magnitude of response according to cell type.

Cohen's revised model of apoptosis would divide the cell death into three distinct mechanisms: induction, release and transduction. Each of these mechanisms describe a cell death that is unique - for example, cells could be described as dying by suicide, murder or execution.

i) induction - the cell initiates *de novo* synthesis of its apoptotic proteins. These cells would be protected if protein synthesis were interrupted (*suicide*).

ii) release - cells that do not require new protein synthesis may be using inhibitors to repress the apoptotic proteins that are already present in the cell. These cells would undergo apoptosis if inhibitor synthesis were interrupted, and can be considered *primed* because they have already undergone the early steps involving protein synthesis at some earlier stage of their differentiation (*murder* - these cells are

prevented from saving themselves).

iii) transduction - cells that would normally require protein synthesis before apoptosis can be *pushed over the edge* if the missing requirements are supplied by another cell. For example, cytotoxic T lymphocytes deliver a lethal package to the target cell by means of a secretory granule that contains perforin (a pore forming protein) as well as other proteases. Several of these have been found to be capable of triggering apoptosis in the target cells (*execution*).

If it was possible to identify the full complement of genes that are necessary for the expression of these different apoptotic programs and recognize the factors that control this expression, it would provide us with powerful new tools to manipulate the process for therapeutic gain of tumor response.

1.6 FUNDAMENTAL COMPONENTS OF THE "DEATH PROGRAM"

Once the death program is initiated it appears to involve a standard set of effector events. The limited evidence available suggests that intracellular calcium in the apoptotic cell may trigger a series of secondary events[44] . Calcium levels have been shown to rise from around 200 nM to sustained levels of about 800 nM in cells undergoing apoptosis[15] . In some cells free cytosolic Ca^{+2} can initiate morphological changes presumably through the activation of calcium dependent transglutaminases and endonucleases. The

transglutaminase proteins have been found to be responsible for the cytosolic protein crosslinking[45], helping to stabilize the apoptotic bodies to limit leakage into the extracellular space. The calcium/magnesium dependent endonuclease *Nuc 18* may be responsible for the characteristic DNA fragmentation described as a *DNA ladder*[46].

An unresolved issue is whether specific genes exist, whose expression regulates this process of endonuclease digestion. For example, the necessity of calcium flux would suggest that the calcium dependent protein kinase C signalling pathway may be involved in transducing the apoptotic message in those cells that respond by increasing intracellular calcium levels. Once again, this can only be assumed to be dependent on cell specific machinery since it is also known that some cells can fragment their DNA without the involvement of calcium. It has also been found that other nucleases can be activated when the cell loses its attachment to the underlying surface. In this case, the Na+/H+ antiporter on the plasma membrane becomes inactivated leading to intracellular acidification and another endonuclease (deoxyribonuclease II) becomes activated[47].

1.6.1 How the Signal is Sent : Proposed Factors Involved

Ultimately the process of apoptosis must be a modulated phenomenon, with inhibitors and initiators acting both intra- and intercellularly. Several putative genes have been identified that when considered together could complete a simplified version of the proposed apoptotic pathway previously considered by Carson[41]. The products of these genes encompass the

necessary components of this model including extracellular factors, membrane surface receptors, cytoplasmic regulators, substrate specific kinases, and nuclear proteins.

i) Cellular Adhesion Factors.

The phenomenon of apoptosis being present in scattered cells within a tissue may be understood by examining the cell-cell interactions. It is well known that cell adhesion mechanisms have been linked to cell survival and cell cycle control[2]. Cells exposed to cytotoxic stimuli can produce diffusible factors that enhance the survival or proliferation of the neighboring cells. Tomei[7] suggests that the apoptotic cells are not only producing this factor, but are also communicating through gap junctions since agents believed to inhibit apoptosis are also known to regulate adhesion receptors. In addition, stimulation of the cell cycle is associated with changes in cell adhesion and this in turn can modulate apoptosis.

ii) Membrane Receptors with Kinase Activity.

Fas is a transmembrane protein related to a family of surface receptors which includes tumor necrosis factor receptor (TNFR) and is known to be expressed in various T and B cell lines, fibroblasts and lymphocytes. Perhaps what is most interesting is that *Fas* antibodies induce apoptosis in these cells at a rate which is much faster that that caused by depletion of growth factors[48]. This suggests that the *Fas* protein may be actively transducing the death signal while a decrease in growth factor may involve an indirect process.

Mutation studies by Itoh *et al.*[48] have suggested that there is a regulatory element in the cytoplasmic C terminus of the TNFR and that tyrosine phosphorylation can inhibit its tyrosine kinase activity, similar to other surface receptors such as EGFR and PDGFR. These other receptors are known to be G protein coupled and contain a phosphorylation site for cAMP dependent kinase in their cytoplasmic domain. The C terminal of *Fas* may regulate a *Fas*-mediated apoptotic signal by a similar mechanism.

iii) Cytoplasmic Regulators: Guanine Nucleotide Binding Proteins.

In 1987 Wyllie[49] found that the active form of the *ras* oncogene product can be involved in many processes including the apoptotic response. High expression of the mutated form can protect the cell from death. In normal cells members of the *ras* family of proto-oncogenes have been highly conserved throughout evolution and produce a 21kd protein known as $p21^{ras}$ that plays a significant role in signal transduction. In response to external stimulation p21 exchanges GDP for GTP and is converted to an *active form*. The activated p21-GTP can then bind an effector resulting in the generation of a number of cellular changes which may include modulation of the apoptotic response.

iv) Serine Threonine Specific Protein Kinases.

Activated *ras* proteins stimulate a group of serine/threonine specific kinases which in turn may directly regulate the transcriptional and translational control of gene expression[50]. In studies with hematopoietic cells it has been found that when a cell is stimulated to die by apoptosis (CTL killing) there is a rapid activation of a kinase called $p34^{cdc2}$ [51]. This kinase is known to play a

central role in the control of the cell cycle, and is critical for the passage from G_2 to M phase.

The activation of the apoptotic response is again very rapid in contrast to the time required to detect activated p34 in its cyclin dependent function at the G_2/M checkpoint. Shi *et al.*[51] suggest that p34 is being *uncoupled* from its normal function. Premature activation of p34 may actually be a consequence of the cell losing control of its cell cycle (for example, other genes such as *c-myc* and *p53* can cause the cell to cycle inappropriately). *p34 c*ould be considered a convergence point for different apoptotic signals. This could explain why different apoptotic stimuli (membrane damage or DNA damage) result in signals being conveyed via the nucleus (presumably by phosphorylation changes to target molecules) ultimately resulting in nuclear dissolution and cell fragmentation.

v) Nuclear Proteins and Transcription Factors.

In response to an apoptotic stimulus it has been found in some cells that there is a cascade like induction of *c-fos* , *c-myc* and *hsp 70*[52]. It is difficult to ascertain whether these new transcripts are integral to apoptosis because they may also be important to other stress responses. Nevertheless the expression of *c-myc* (a known nuclear transcription factor) does seem to have a regulatory effect, determining either proliferation or apoptotsis depending on the availability of critical growth factors.

If cells are modified to express *c-myc* constituitively (as is often the case in cancer cells) the withdrawal of growth factors will not result in a growth arrest and the cells will remain in cycle. But only some of the cells will successfully

complete DNA replication, for a substantial number of them will die by apoptosis[53] . The mechanism by which *c-myc* regulates this process is unknown and once again there is no firm conclusion that can be drawn for all cell types.

Although the apoptotic signalling network remains to be delineated, there is no doubt that the response of an apoptotic cell culminates in the digestion of its genome. The DNA ladder fragmentation has long been considered to be the definitive event of the process, but this may actually be a late event .

At the present time there is agreement that events within the nuclear membrane precede any nucleosomal cleavage. There exists an intranuclear framework analogous to the cytoskeleton on which the nuclear components are organized. The characteristic reorganization of the chromatin just under the nuclear membrane prior to its disintegration may be due to specific proteins within the nuclear scaffold binding to specific DNA sequences. This event may be very important to the subsequent cleavage of the DNA and may in fact be a better indicator of apoptosis. Therefore the determination of this nuclear reorganization event should be included in any morphological assay.

Apoptotic fragmentation patterns seen with gel electrophoresis suggest that DNA is broken down in at least two steps involving at least two different endonucleases. Characterization of the nuclease(s) involved in apoptotic DNA cleavage has not been determined although Arends *et al.*[6] have suggested that an endonuclease *Nuc 18* may be responsible for the 180 integer base pair fragmentation in thymocytes. Filipski *et al.*[35] propose that there are 5 levels of folding in DNA that must be systematically dismantled during apoptotic fragmentation. This process is thought to follow a distinct pattern of endonucleolysis which results in fragments from approximately 9 mbp (chromatin

coils) to 300 kbp (rosettes) to 50kbp (loops) and then finally to the ladder fragments consisting of nucleosomes of 146 base pairs with a histone core, separated by a region of about 60 base pairs.

Studies of the endonucleolytic process in many different cell types have revealed fragmentation characteristics corresponding to the first three levels of chromatin organization. Oberhammer *et al.*[54] compared the morphological and biochemical features of cell death in several epithelial carcinoma cell lines and one rat transformed fibroblast line. Using field inversion gel electrophoresis, cleavage to 300 and/or 50 kb fragments was detected prior to or in the absence of internucleosomal fragmentation.

vi) Tumor Suppressor Genes: *bcl2* and *p53*.

The *bcl2* proto-oncogene may be termed a generalized cell death suppressor. All hematopoietic and many epithelial cells contain this protein which is found mainly in the mitochondrial membrane, nucleus and endoplasmic reticulum of the cell. It is the deregulation of this gene that is found in follicular B cell lymphoma (hence the name *bcl2*). There is an increase in the amount of Bcl2 protein due to a t(14:18) translocation that places the regulation of the gene under control of the immunoglobulin heavy chain promoter. In these cells the high expression of *bcl2* suppresses apoptosis [55] .

Walton *et al.*[56] suggest that the association of the Bcl2 protein with the mitochondrial membrane raises the possibility that the protein may in fact be interacting with the intracellular ATP metabolic process, pH regulation or calcium homeostasis. All these cellular functions have been found to be somehow

related to the apoptotic response in that energy is required and regulation of endonucleases have been linked to pH and calcium fluctuations.

Recently, Willingham *et al.*[57] reported mitosis specific localization of *Bcl2* in human carcinoma cells suggesting a mechanism by which the physiological properties of *bcl2* might affect the apopototic process by directly affecting the nuclease fragmentation of the cell DNA :

> " The specific expression of *bcl2* at the beginning of mitosis, before the dissassemby of the nuclear envelope barrier and its disappearance in telophase, when the barrier reappears, suggest that *bcl2* attached to chromosomes serves a specific function during the time that the chromosomes are accessible to the cytoplasm. The most obvious interpretation would be that *bcl2* serves a protective role, preventing nuclease attack at internucleosomal sites." [Willingham and Bhalla, (1994) p 446-447.]

p53 is a gene product that appears to act via two different mechanisms in regulation of apoptosis. First, in response to DNA damaging agents wild type *p53* can induce temporary G_1 arrest in some cells[58]. It is possible that this arrest cooperates with the endogenous *c-myc* expression to inititate apoptosis. In addition, *p53* can act as a direct *apogene* since its overproduction can induce rapid cell death. The different cellular responses (G_1 arrest vs apoptosis) may result from the activation of distinct target genes by *p53* or alternatively activation of the same target genes in two cell types could have different consequences[59]. In any event, *p53* seems to be able to prevent the accumulation of genetically damaged cells by activating the apoptotic program once the damage is too extensive to be repaired and as such, is referred to as a tumor suppressor.

vi) DNA Damage Recognition Factors.

Many enzymes have been characterized to act on DNA or that use DNA as a template but very few have been described that actually use DNA as a signal to act on something else. Anderson[60] has described a nuclear serine/threonine protein kinase which is activated *in vitro* by DNA double stranded termini. This factor is termed DNA-dependent protein kinase (DNA-PK). The kinase substrates of DNA-PK are also nuclear, and include sp1, Fos, Jun, Myc, p53 *and* RNA polymerase II. The fact that DNA-PK seems to be phosphorylating regulatory components involved in transcription suggests that it may be involved in activating genes whose products function in bringing about G_1 checkpoint arrest, DNA repair or triggering of apoptosis[61]. In the case of apoptosis triggering, *p53* may be an important player. If the phosphorylation of this protein by DNA-PK led to increased expression or enhanced stabilization, this could result in enhanced survival or cell death depending upon which of the various post irradiation pathways a cell chooses to follow.

p53/DNA-PK dependent and independent pathways may share common elements that require protein synthesis or phosphorylation regulation downstream, for example substrates such as the apoptotic nucleases and cytoplasmic transglutaminases. Alternatively, apoptosis can be triggered downstream of p53 in these cells.

1.7 APOPTOSIS INDUCTION AGENTS: IONIZING RADIATION AND PHOTODYNAMIC THERAPY

1.7.1 Ionizing Radiation.

For many years the effect of radiation on cells has been measured by cell survival assays, which determine the extent to which the population of treated cells is able to produce colonies. Survival is then plotted on a log scale as a function of dose on a linear scale. Each distinct cell population taken from a tumor is assumed to have unique molecular and biochemical characteristics resulting in a unique *survival curve*. The extent to which this curve is sloped is an indication of the inherent radiosensitivity of that cell line.

Many different mathematical models have been proposed to define the shape of these curves and may be useful to predict the probability that an individual cell will survive a certain dose of radiation, which in turn may help to predict how a tumor will respond to radiotherapy.

Apoptosis may be an important factor in this prediction. Studies with murine tumor cell lines have shown there is a time and dose response of these cells to radiation induced apoptosis[62]. The *time* response was found to peak at 4 hours and was independent of the dose which covered a range of 2.5 to 25 Gy. This is consistent with the idea that radiation is a trigger for the apoptotic process that once initiated proceeds with kinetics characteristic of the cell type. The *dose* response curve appeared to plateau at about 7.5 Gy and doses of 2.5

Gy were the most efficient for induction. If this apoptotic response could be reliably modelled, it may be possible to predict a tumor's treatment response with even greater accuracy.

1.7.2 Photodynamic Therapy.

Photodynamic therapy (PDT) is a cancer treatment based on the accumulation in malignant tissue of a photosensitiser with low systemic toxicity. Subsequent illumination of the tissue induces a photochemical reaction in which singlet oxygen and other free radicals are produced, resulting in the destruction of various macromolecular cell components. In the clinic the effect of PDT can be manipulated as the light source (argon dye laser) can be tuned to an appropriate wavelength and delivered either superficially, interstitially or intraluminally[63]. The effects of PDT depend in a complex way on: 1) tissue concentration and localization of the photosensitizer; 2) the target tissue optical properties and oxygenation; 3) activation wavelength; 4) power density and 5) treatment regimen.

The laser can be coupled to one or more fibre optic cables to propagate light with minimum energy loss to a tip. The amount of energy delivered will depend on the duration of the light delivery and the dose rate of the light[63]. Perhaps what is most interesting when considering apoptosis and PDT is that lowering the dose from 200 - 50 W/cm shows an enhanced PDT response *in vitro*. Although it was initally hypothesized that reduction in the fluence rate, or fractionation of the light dose, may increase the effective singlet oxygen dose in regions of sparse capillary density[64] it could be argued that this lower light dose is triggering apoptosis in the mouse tumor cells since low doses of γ irradiation

have been shown to induce apoptosis in murine tumors[62] . The use of the current clinically approved photosensitizers available in the clinic has caused some concern for the toxic effects on normal tissue. For example, patients treated with hematoporphyrin derivatives can develop symptoms such as erythema, edema and pain in light exposed areas. Cutaneous photosensitivity arising from hematoporphyrins can remain for several months but is usually 6 - 8 weeks in duration[65]. New sensitizers are now being developed with a view toward an enhanced photodynamic effect, utilization of simpler light sources and elimination of concurrent skin photosensitization[63].

Hypocrellins A (HA) and B (HB) are two main pigments isolated from the parasitic fungi *Hypocrella bambusae Sacc* and *Shirai bambusicola P.* that are found in China. These pigments have a long history as traditional medicinal agents[62]. Two derivatives of the parent compound Hypocrellin B being developed at the University of Alberta/Cross Cancer Institute for use in photodynamic therapy include HBEA-R1 and HBBA-R2[67].

II EXPERIMENTAL OBJECTIVES

2.1 WHAT IS THE BEST WAY TO ASSAY FOR APOPTOSIS IN HUMAN TUMOR CELL LINES: DO THE QUALITATIVE AND QUANTITATIVE MEASURES FROM EACH ASSAY CORRELATE?

Within the context of our *in vitro* human tumor model, we wished to determine 1) whether apoptotic heterogeneity existed between and within tumors, and 2) if different assays for apoptosis yielded comparable results. The assays chosen were flurorescence microscopy, agarose gel electrophoresis, zero integrated gel electrophoresis and flow cytometry. Since each of these procedures has its own inherent limitations, we also wished to determine which method or combination of methods may be most useful for measuring apoptosis in human tumor cells both qualitatively as well as quantitatively.

2.2 DOES INTER- AND INTRATUMORAL HETEROGENEITY EXIST WITH RESPECT TO APOPTOSIS SUSEPTIBILITY?

Malignant gliomas are known to be histologically pleomorphic and genetically unstable. Differences in the apoptotic response between two cell lines derived from the same tumor would suggest possible alterations in the regulatory pathways. It has been previously documented that gene expression of some tumor suppressor genes such as *p53* and *Rb* differ at the level of RNA within and among these glioma cell lines [64]. Does this heterogeneity extend to the induction of apoptosis?

2.3 COULD THE PATHWAYS INVOLVED IN APOPTOSIS AND RADIATION SENSITIVITY BE SIMILAR?

A good correlation has been previously reported between spontaneous levels of apoptosis in unirradiated tumors and the extent of radiation induced apoptosis in murine tumor cell lines [69]. If this could be shown in human tumors it may hold promise for a future predictive assay for response to radiotherapy. It has also been proposed that the radiosensitivity of a cell line correlates with its suseptibility to apoptosis. Human cell lines which exhibit significant differences in sensitivity to radiation may be useful models to examine the link to radiation response. The human malignant glioma cell lines M016, M071, M059J and M059K developed by Allalunis-Turner *et al.*[70] at the Cross Cancer Institute, Edmonton, are among several cell lines that have been characterized for their inherent sensitivity to ionizing radiation and chemotherapeutic agents. Two of these lines, M059J and M059K, were established concurrently from a single tumor specimen and are approximately 30-fold different in their radiation sensitivity as measured by surviving fraction at 2 Gy (SF2; M059J = 0.02, M059K = 0.64). The cell lines M016 and M071 were chosen for study as they are representative of relatively radioresistant (M016, SF2 = 0.68) and radiosensitive (M071, SF2 = 0.19) glioma cell lines.

Because these four cell lines differ in their radiosensitivity, they provide a good model in which to evaluate the contribution of apoptosis to overall radiosensitivity. In addition, because M059J and M059K cells were derived from the same biopsy specimen, they provide a good model in which to determine whether intratumoral differences in apoptosis exist.

2.14 IS APOPTOSIS AN IMPORTANT MODE OF CELL DEATH IN PHOTODYNAMIC THERAPY?

Drug uptake studies with confocal microscopy have shown that the photosensitizing drugs HBEA-R1 and HBBA-R2 are concentrated in the cytoplasmic regions of the cell [67]. Does the oxidative stress presumed to occur after laser illumination induce membrane damage which is capable of triggering the apoptotic process? The signal transduction pathways that inititate apoptosis in PDT remain undefined. However, it is possible to evaluate the incidence of apoptosis in tumor cells treated with photosensitizing agent only (dark) or treated with photosensitizing agent followed by laser illumination (light). The propensity of a cell line to undergo apoptosis after a photosensitizer drug and illumination compared to the apoptotic propensity of drug treated cell alone may be clinically important in that it may estimate the efficacy of this therapy.

III MATERIALS AND METHODS

3.1 RADIATION INDUCED APOPTOSIS IN HUMAN GLIOMA CELLS.

3.1.1 Cell Lines, Inherent Radiosensitivity, and Genetic Status

Portions of diagnostic biopsies obtained from patients with malignant glioma were established as primary cultures as described previously by Allalunis-Turner *et al.*[70] Cultures were maintained by serial passages in DMEM/F12 medium (1:1 ratio) supplemented with 10% fetal bovine serum. Inherent radiation sensitivity of the four cell lines used have been previously documented [70]. Values for the surviving fraction at 2 Gy (SF2) were as follows: (M016 = 0.68), (M071 = 0.19), (M059K = 0.64) and (M059J = 0.02). These SF2 values were determined from the best fit curves obtained using the linear quadratic model. There was no significant difference between the calculated and measured SF2 values.

Table I is a summary of the information obtained in previous experiments regarding the status of the *p53, Rb, and DNA-PK* expression in the four glioma cell lines.

Table 1

Genetic Status and SF2 values - M016, M071, M059J, M059K

CELL LINE	SF2[70]	p53[68]	Rb[68]	DNK-PK[71,72]
M016	0.68	-	++	18.0
M071	0.19	+	++	4.4
M059J	0.02	+++	-	0.03
M059K	0.64	+++	-	6.3

Levels of p53 and Rb mRNA expression: ++ = average expression, +++ signifies about 2 fold greater than average expression. The presence or absence is indicated by '+' or '-'. DNA-PK activity measured as nmol/min/mg protein.

3.1.2 Irradiation Procedure

Cells were trypsinized and replated at a density of approximately 2 x10^6 per 75 cm flask (or as specified by the procedure), placed in an incubator at 5% CO_2 /balance air, 37°C and allowed to grow for 24 hours. These cells were then irradiated at room temperature using a γ – ray source, either ^{137}Cs (1.72 Gy/min) or ^{60}Co (5.5 Gy/min). Doses were as follows : 0, 2.5, 5 and 10 Gy. Following irradiation, the cells were returned to the incubator for various times (0, 6, 24, 48 hours) prior to assaying for apoptosis.

3.1.3 Fluorescence Microscopy.

Cells were trypsinized from the flask and added to the nonadherent floating cells within the media at appropriate times after irradiation and spun onto slides with a cytocentrifuge (Shandon Cytospin 3), $2x10^4$ cells/slide. The slides were then fixed in acetone for 15 minutes and stained at 4°C with propidium iodide (10 mg/ml) for another 20 minutes. Using a Leitz Laborlux microscope (530 nm filter), the cells were examined for apoptotic morphology. The appearance of condensed nuclei (previously described) was used as evidence of apoptosis in control and irradiated cells. The % apoptotic cells per slide was ascertained and was based on counting no less than 400 cells per slide and three slides per time period. Photomicrographs typical of the appearance of apoptosis in each cell line were obtained.

3.1.4 Conventional Agarose Gel Electrophoresis.

Cells were seeded and irradiated as described for microscopy studies. At various times after irradiation, DNA was extracted from the combined attached and detached control and irradiated cells using a commercially available extraction kit (Stratagene). After aquiring total DNA, the lysates were centrifuged at 27,000 g for 20 minutes to separate the low molecular weight DNA (indicative of apoptotic fragmentation) as per Langley *et al.*[73] The DNA samples were then treated with RNase (20 µg/ml) and incubated for 15 minutes at 37°C . After extraction with 100% ethanol, the DNA was washed briefly with

70% ethanol and carefully resuspended in 500µl of 10mM Tris, 0.1 nM EDTA buffer and stored at 4°C.

The DNA samples were separated by gel electrophoresis (1% agarose gel, 72 volts for 4 hrs) and visualized under UV illumination after staining with ethidium bromide. A photographic record of each gel was obtained using a Polaroid camera system.

3.1.5 Zero Integrated Field Gel Electrophoresis.

Cells were seeded (as above) and allowed to grow 24 hours. The cells were then labeled with ^{14}C-thymidine (specific activity of 0.53 mCi/mM, diluted to a final concentration of 0.03 µci/ml) for 24 hours prior to irradiation. Immediately after radiation treatment the cells were trypsinized and added to the detached media cells, spun down and placed into agarose plugs ($1x10^6$ cells/plug), then re-incubated at 37°C. After the specified times, the cells were set into lysis buffer (0.5 M EDTA pH 8, 0.01 M Tris, 2% sodium-N-laurylsarcosine , 0.1 mg/ml proteinase K and 100 mls distilled water) for 12 hrs. The cells were then washed in TE buffer (.1M EDTA pH8, 0.01 M Tris) for one hour (37° C) before adding RNase (20µg/ml) and allowing the cells to incubate for an additional hour.

The preformed plugs were then loaded into the wells of a 1% agarose gel and run on a computer controlled zero integrated field gel electrophoresis system (ZIFE) (Q-LIFE GenePak Autobase System). The computer analysis programs used pre-set ROM cards (card numbers 3 and 5) to examine DNA fragments (ranging from 8 - 500, and 100 - 1100 kbp respectively) by controlling

the run conditions (forward and reverse voltage x duration). These size distributions were chosen to include the range of fragment sizes previously described as being characteristic of apoptotic fragmention in other solid tumor cell lines [67]. Molecular weight and size standard markers (Megabase II Standard DNA 48.5kb - 1.3mb, GIBCO, and High Molecular weight DNA 9 - 48kb, GIBCO) were run concurrently.

Upon completion of the run, the gel was stained with ethidium bromide, visualized and photographed under UV light, and then cut lane by lane into 2 cm pieces. Because the cells had been previously radiolabelled with ^{14}C-thymidine, the counts per block could be determined with a scintillation counter. Each of the 2 cm blocks of agarose corresponded to a small range of fragment sizes. These counts were then plotted as a function of the distance the DNA migrated into the gel. Calculating the % total DNA in the lane, it is possible to quantitatively estimate the amount of DNA that is fragmented to the size indicative of apoptosis (approx 300 kbp). This in turn gives an indication of the % apoptotic cells in that population following a given period of time.

3.1.6 Flow Cytometry.

Control and irradiated cells were trypsinized, added to the nonadherent media cells, and prepared for flow cytometric analysis using protocols established by Vindelov *et al.* [73] Briefly, cells were resuspended in 200 µl of citrate buffer (250 mM sucrose, 40 mM trisodium citrate and 5 ml/100ml dimethylsulphoxide - pH adjusted to 7.60) and allowed long term storage at -80°C The stain solutions were prepared as follows:

Stock solution: trisodium citrate (3.4 mM), nonidet P 40 (0.1%), spermine tetrahydrochloride (1.5 mM), Tris (0.5 mM) - pH adjusted to 7.6.

Solution A: 15mg trypsin (Sigma) dissolved in 500 ml stock solution - pH adusted to 7.6

Solution B: Trypsin inhibitor (Sigma) and ribonuclease A (50 mg) added to 500ml stock solution - pH adjusted to 7.6.

Solution C: Propidium iodide (208 mg), spermine tetrahydrochloride (580 mg) added to 500 mls of stock solution - pH adjusted to 7.6.

At the appropriate times the cells were stained with the above solutions according to the following protocol: i) 900 µl solution A with gentle inversion for 10 minutes ii) add 750 µl solution B with gentle inversion for 10 minutes iii) add 750 µl solution C with gentle inversion for 15 minutes. The cells remained in this combined solution for 12 hours prior to flow cyt metric analysis .

Normal human lymphocytes served as diploid control and were prepared as were the tumor cells. This was to ensure that the staining procedure was effective. The DNA distribution was then analyzed using a FACScan flow cytometer. The CellFIT program was used for data aquisition and the initial detection of apoptosis was indicated by a shift in the G1 population[32] . The proportion of apoptotic cells was quantified using the LYSIS II analysis program.

3.2. PHOTODYNAMIC THERAPY-INDUCED APOPTOSIS.

3.2.1 Cell Preparation.

EMT-6 cells were grown in monolayer using Waymouths media with 13% fetal bovine serum. After incubation at 37°C for 24 hours, the cells were trysinized, washed, resuspended, counted before replating onto 35 x 10mm culture dishes at the following densitites : $5x10^4$, $1x10^5$ or $5x10^5$ cells/plate.

These plating densities were chosen to account for the fast doubling times of these cells and also the large amount of cell death that occurs upon PDT treatment .

3.2.2 Drug Treatment

After seeding the cells were returned to the incubator for 24 hours at 37° . Following incubation, 40 µl of either HBEA-R1 or HBBA-R2 in pure DMSO (0.20 µM solution) was added to the cultures for a total exposure time of 2 hours at 37°C. From the time of drug addition the cells were handled in subdued light to avoid extraneous light activation of the photosensitizer drugs.

3.2.3 Laser Treatment

Photosensitizing agents were removed by washing the plates twice with 1 ml of Hank's buffered saline solution, leaving the final wash (1 ml) on the plates. The control and "dark" samples were left covered and the "light" treated samples were treated with 1 Joule/cm^2 of 630nm light (60 sec x 160 mW). The laser specifications are as follows:

-Argon pumped dye tunable laser (Coherent Innova Model CR599 dye laser with Kiton Red (Exciton, Dayton Ohio) as the laser dye. The 630 nm

monochromatic light was focused into a 400 micron diameter quartz optical fiber with microlens on the output end. The fibre is positioned 9.7 cm above a rotating stage to allow for even illumination with the spot size of 36mm (same dimensions as the dish).

3.2.4 Apoptosis Assays

At the appropriate times after treatment (4, 24, 36, 48 hours) the cells in dishes were removed from the incubator, spun onto slides and stained with propidium iodide (as previously described for glioma cells), and examined under the fluorescent microscope for evidence of condensed nuclei characteristic of apoptosis. The percent apoptosis was determined by counting a minimum of 200 cells on 6 slides per time interval. Results were expressed as mean ± standard deviation.

In other experiments, DNA was extracted from EMT6 cells which had been treated with HBEA-R1 or HBBA-R2 and incubated in the dark for a specified time (dark samples) or illuminated with the laser and then incubated for the specified time (light samples). Conventional agarose gel electrophoresis was perfomed in order to determine whether EMT-6 cells fragmented their DNA to the 180 bp multimer - DNA ladder. When these results suggested that endonucleolysis was terminated at larger fragment size, ZIFE was used to determine the time of appearance of apoptosis as well as the fragmentation characteristics of the DNA. The procedures used for both electrophoresis gels were the same as previously described for glioma cells and radiation induction of apoptosis.

IV RESULTS

4.1. RADIATION INDUCED APOPTOSIS.

4.1.1 Fluorescence Microscopy.

Control and irradiated cells were examined at 0, 24 and 48 hours post irradiation (10 Gy) for evidence of apoptosis. As shown in figure 1, DNA fragmentation characteristic of apoptotic cells[1] was observed in all cell preparations, both as a spontaneous event (in controls) as well as an induced event in the irradiated cells. The visual pattern of DNA fragmentation included marginated condensed chromatin and bead-like structures within the nucleus. Quantification of the percent of apoptotic cells/slide is shown in Table II. The results indicate that in all cell lines, cells with DNA fragmentation characteristic of apoptosis were present at low numbers in unirradiated samples. Following exposure to 10 Gy, the percentage of apoptotic cells increased in all cell lines. However, only in M059J were the mean values significantly different from the background control values.

Figure 1

Photomicrographs of Glioma Cells Stained with Propidium Iodide after Radiation Treatment

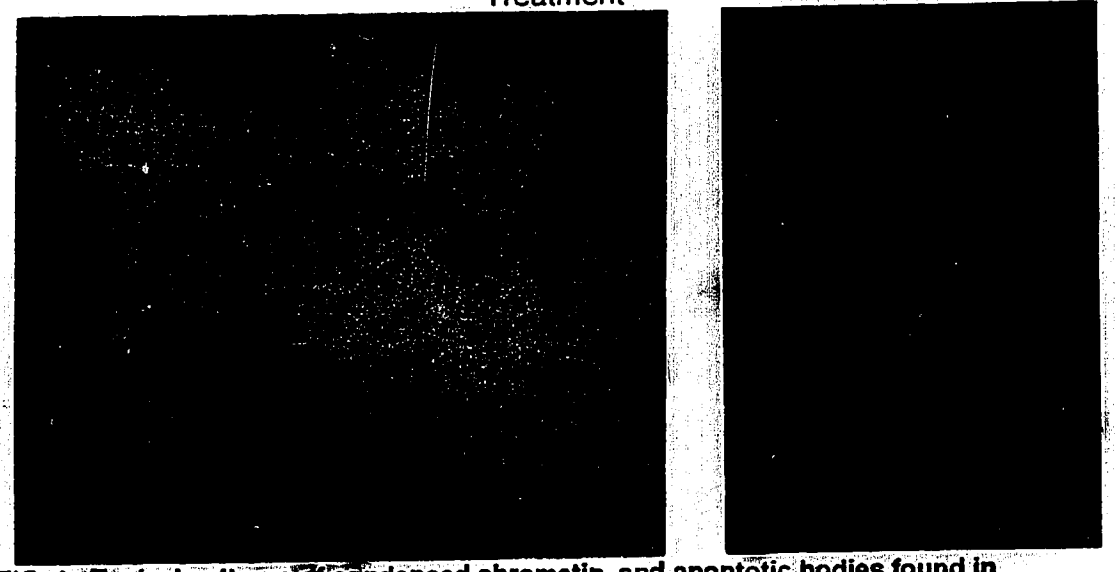


FIG. 1. Typical patterns of condensed chromatin and apoptotic bodies found in MO59J and M059K cells 24 hours after 10Gy irradiation are shown. Cells were stained with propidium iodide. 500X magnification.

Table II

Fluorescence Microscopy - Percent Apoptotic Cells per Slide

Cell line	Control	24hrs after 10 Gy	48hrs after 10 Gy
M016	10 ± 3%	11 ± 3%	20 ± 7%
M071	9 ± 4%	9 ± 2%	15 ± 2%
M059J	12 ± 4%	19 ± 2%	22 ± 4% **
M059K	15 ± 4%	13± 3%	18 ± 4%

* These measurement represent mean ± standard deviation based on three slides per cell line.

**p<.035.

4.1.2 Gel Electrophoresis

Conventional gel electrophoresis was used in an attempt to determine whether the DNA fragmention observed with the microscopic analysis would produce a pattern characterisitic of DNA laddering. DNA was extracted from each cell line at 24 and 48 hours after 10 Gy and was run on an agarose gel. Molecular weight standards were included in lane 1. Photographs of ethidium bromide stained gels are shown in figure 2. DNA extracted from the irradiated cells migrated as a single band. The *classic* laddering pattern was not observed suggesting that the 180 integer fragmentation did not occur in these cells.

Figure 2

Conventional Gel Electrophoresis for Apoptotic DNA Fragments

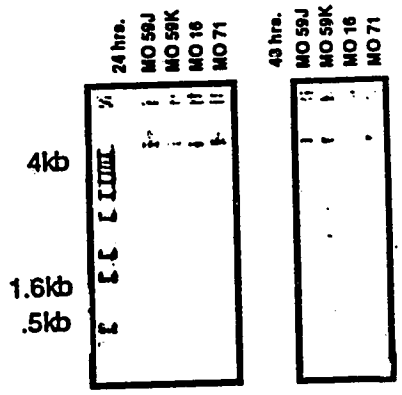
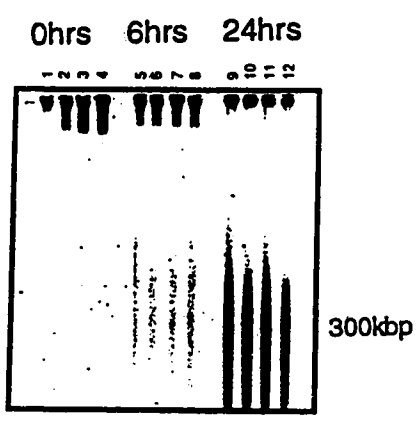


FIG 2. Agarose gel electrophoresis of DNA extracted from human glioma cells after 10 Gy irradiation. Cells were collected after 24 hours (lanes 3-6) and after 48 hours (lanes 7-10). 1 kb molecular weight markers were run simultaneously (lane 1).

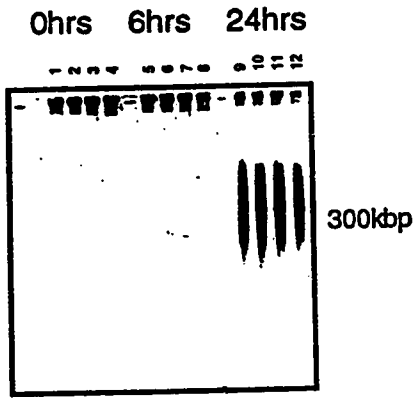
Zero Integrated Field Electrophoresis (ZIFE) was used to delineate the size of the DNA fragments produced by radiation treatment. Using Q-LIFE pre-set ROM cards, a computer analysis program was set to examine fragments ranging from 8-500 kbp (card 3) and 100-1100 kbp (card 5). Figure 3 shows the photographed agarose gels containing M016, M071, M059J and M059K DNA respectively. The majority of the fragments were approximately 300 kbp in size at 24 hours.

Figure 3

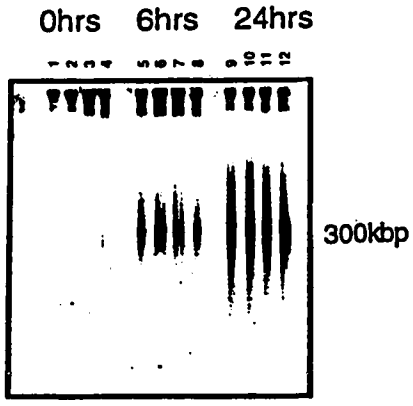
Zero Integrated Field Gel Electrophoresis Analysis of Apoptotic Fragmentation



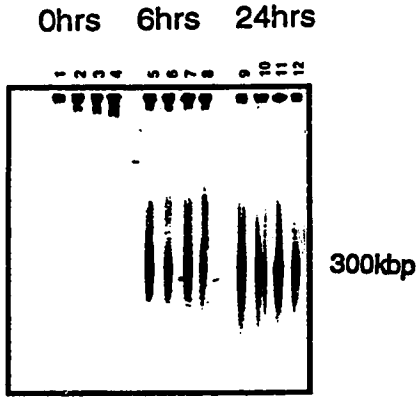
M016



M071



M059J



M059K

FIG 3. ZIFE of DNA from whole cells , attached and detached, after 0 hours, 0, 2.5. 5 and 10 Gy (lanes 1-4) 6 hours, 0, 2.5, 5 and 10 Gy (lanes 5-8) and 24 hours 0, 2.5, 5 and 10 Gy (lanes 9-12). Molecular weight standards were included in each gel in the leftmost lane.

ZIFE was also used to determine the pattern of DNA fragmentation at various times after irradiation. Figure 4 shows the apoptotic induction in all four cell lines 6, 24 and 48 hours after 10 Gy. These results suggest that all four cell lines show the greatest percentage of apoptotic fragmentation after 48 hours. Scintillation counts of the DNA fragments showed a major peak at approximately 300 kb as determined by reference to molecular weight markers run simultaneously. The percentage of counts for each cell line that were found within this peak after 0 Gy and 0 hours (control) or 10 Gy irradiation and 6, 24 or 48 hours are shown in Table III. There is relatively little fragmentation occurring in the control cells but over time the DNA is cleaved in a controlled manner until the majority of the fragments are found at the 300 kb size after 48 hours.

Figure 4

Zero Integrated Field Electrophoresis Analysis of Apoptotic Fragments at Various Times Post Irradiation

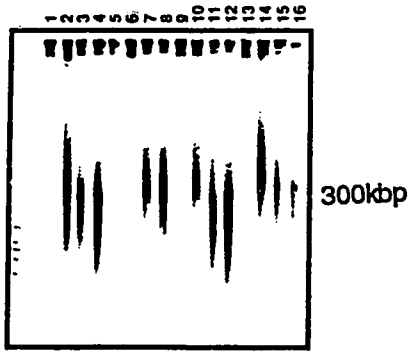


FIG 4. ZIFE of 4 cell lines run simultaneously (combined attached and detached cells). M059J 0 hours and 0 Gy control (lane 1), 10 Gy, 6, 24 and 48 hours (lanes 2-4), M059K 0 hours and 0 Gy control (lane 5), 10 Gy, 6, 24, 48 hours (lanes 6-8), M016 0 hours and 0 Gy control (lane 9), 10 Gy, 6, 24 and 48 hours (lanes 10-12), M071 0 hours and 0 Gy control (lane 13), 10 Gy, 6, 24, 48 hours (lanes 14-16).

Table III

ZIFE Scintillation Counts of Apoptotic Fraction Expressed As Percentage of DNA in the Lane at Various Times After Irradiation

Cell Lines	Control Ohr	6hr (10 Gy)	24hr (10 Gy)	48hr (10 Gy)
M016	0	0	4	11
M071	0	4	9	10
M059J	0	3	13	28
M059K	0	6	11	14

4.1.3 Flow Cytometry.

The DNA content of M016, M071, M059J and M059K before and after irradiation was analyzed using the CELLFIT program. Subsequently the LYSIS II program allowed a quantitative measure of the percentage of cells in the sub G1 G1, S and G2 regions. The percentage of cells within the sub G1 region are considered to represent apoptotic cells which contain fractional amounts of DNA. The histograms shown in figure 5 allow for a qualitative comparison of the four cell lines at 0 hours (control) and 48 hours after irradiation with 10 Gy. The results at 24 hours did not differ significantly from the controls and are not shown. Table IV shows the percentage of cells with a sub-G_1 content after 48 hours. Only MO59J cells showed a G_1 shift to the left, indicative of apoptosis.

Figure 5

Flow Cytometry Histograms - DNA Content in the Sub G1 Region

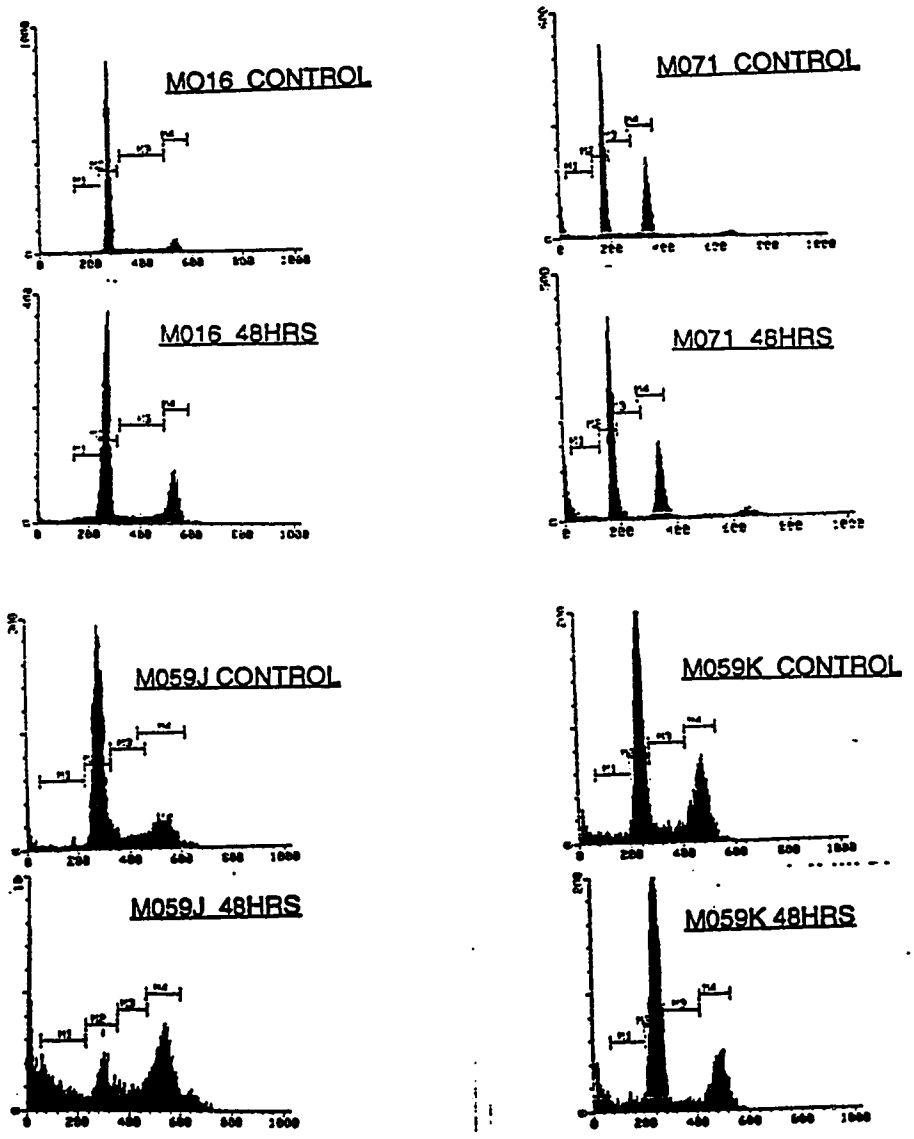


FIG 5. Flow cytometry histograms of DNA content analysis in control (0 hours) and irradiated cells (48 hours) The four cell cycle regions were quantitated within the following boundaries - M1-Sub G1 (apoptotic region), M2-G1, M3-S and M4-G2.

TABLE IV

Flow Cytomety - Percent DNA in Sub G1 (Apoptotic) Region

Cell line	% DNA in Sub G1 Region
M016	7
M071	4
M059K	4
M059J	18

4.1.4 Interpretation of the Percentage of Fragmentation Results From Microscopy, Electrophoresis and Flow Cytometry.

Figure 6 gives a comparison of the results obtained from the three quantitative assays for percent apoptosis 48 hours after 10 Gy. In each case, the most radiosensitive cell line, M059J was the most susceptible to apoptosis induction. In contrast, little apoptosis was observed in the two radioresistant cell lines, M059K and M016.

Figure 7 compares the percent of radiation induced apoptosis as a function of the varying radiosensitivities of the four cell lines. These results suggest that there is no correlation between the inherent radiosensitivity of the cells and their abiity to initiate apoptosis.

Table V

Summary of Estimates of Percent Apoptosis Using
Three Different Quantitative Methods

Cell Lines	Fluorescence Microscopy	ZIFE	Flow Cytometry
M016	20	11	7
M071	15	10	4
M059J	22	28	18
M059K	18	14	4

Figure 6

Radiation Sensitivity (SF2) vs Percent Apoptosis in Glioma Cells

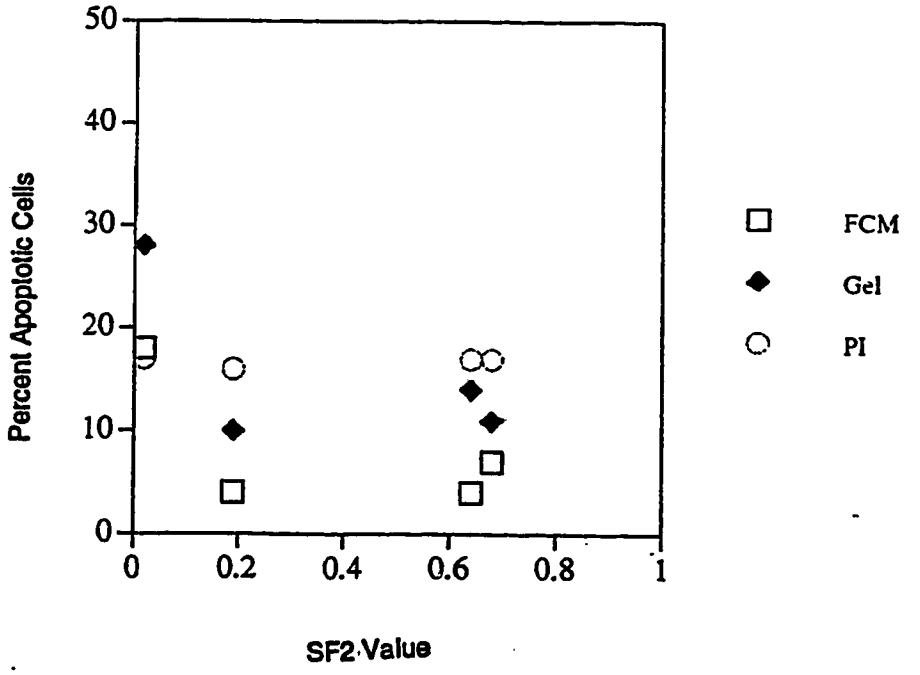


FIG 6. Percentage of apoptotic cells as a function of the their radiosensitivity (as measured by SF2 value). □ = flow cytometric analysis of sub G1 content, ♦= ZIFE analysis of DNA fragments, O= propidium iodide staining of cyto-centrifuge preparations.

4.2 PDT INDUCED APOPTOSIS

4.2.1 Fluorescence Microscopy.

Control, dark treated and light treated cells were examined at 4, 24 and 48 hours after treatment for evidence of apoptotic nuclear condensation, using the same methods described for radiation induced apoptosis. Table V shows the mean ± standard deviation percentage of apoptotic cells in each sample. Photomicrographs of apoptotic EMT6 cells are shown in figure 8.

Table VI

PDT Fluorescence Microscopy - Percent Apoptotic Cells per Slide

TREATMENT	4 hours	24 hours	48 hours
HBBA-R2			
control	2.5 ± 0.7	2.6 ± 0.9	3.4 ± 1.1
dark	3.6 ± 1.3	3.5 ± 1.2	4.9 ± 2.1
light	6.8 ± 0.7	11.6 ± 2.5	30.2 ± 3.5
HBEA-R1			
control	1.4 ± 0.5	n.d.	1.8 ± 0.3
dark	1.6 ± 0.9	2.0 ± 0.7	2.8 ± 0.6
light	2.8 ± 2.2	11.6 ± 2.6	41.7 ± 4.0

Figure 7

Photomicrographs of EMT6 Cells Stained with Propidium Iodide After Photodynamic Therapy

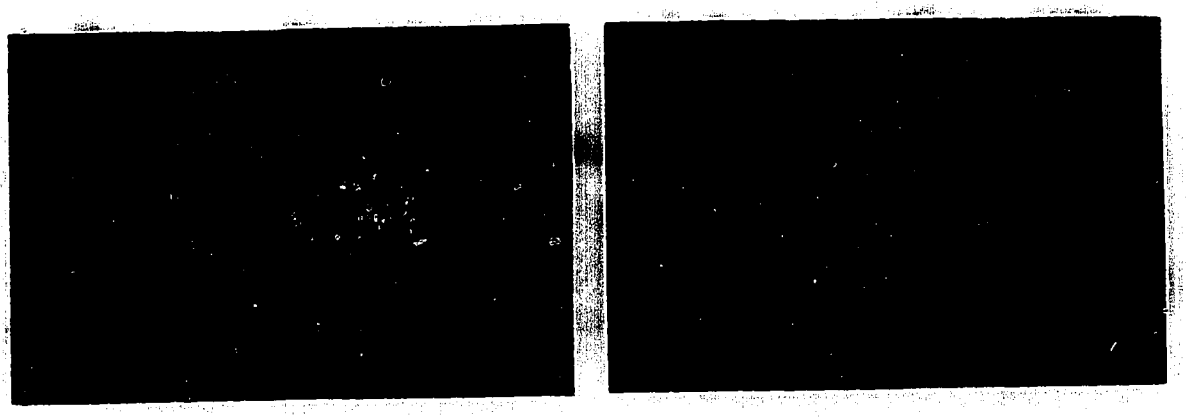


FIG 7. Typical patterns of condensed chromatin and apoptotic bodies found in EMT6 cells 24 hours after PDT treatment. Cells were stained with propidium iodide. 500X magnification is shown.

4.2.2 Gel Electrophoresis

Conventional agarose gel electrophoresis was used to determine if EMT6 cells show the DNA ladder fragmentation pattern. The DNA from the treated cells was shown to migrate as a single band. Figure 8 shows DNA from both dark and light treated cells.

Figure 8

Conventional Agarose Gel Electrophoresis - Analysis of EMT6 DNA after Photodynamic Therapy

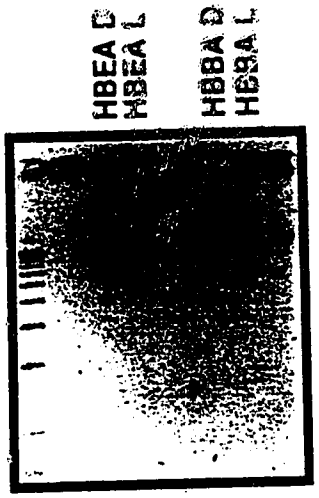


FIG 8. Conventional agarose gel electrophoresis to determine classic DNA laddering. Extracted DNA from cells 24 hours after treatment with HBEA-R1 and HBBA-R2 in the dark (D) or following laser treatment (L) are shown. Molecular weight standards (1Kb) are shown in lane 1.

Zero Integrated Field Electrophoresis (ZIFE) was used to determine the pattern of fragmentation at various times after PDT treatment. Dark treated cells showed low background levels of apoptotic fragmention after 24 hours. Significantly higher levels of fragmentation were found in the cells which had been illuminated with the laser (light treated cells) after 24 hours. Figure 9 shows the fragmentation patterns of both HBEA-R1 and HBBA-R2 treated cells.

Figure 9

Zero Integrated Field Electrophoresis of DNA Fragmentation -PDT Treatment

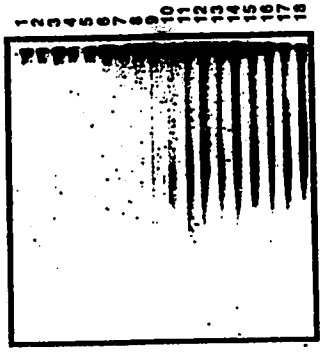


FIG 9. ZIFE analysis of attached and detached EMT6 cells following PDT treatment. HBEA-R1 treated cells after 4 hours , Control , dark and light (lanes 1-3), HBBA-R2 treated cells after 4 hours, control, dark and light (lanes 4-6), HBEA -R1, 24 hours dark and light (lanes 7,8), HBBA-R2 24 hours dark and light (lanes 9,10), HBEA-R1, 36 hours dark and light (lanes 11,12)., HBBA-R2, 36 hours dark and light (lanes13,14), HBEA-R1, 48 hours dark and light (lanes 15,16), HBBA-R2, 48 hours dark and light (lanes 17,18). These results indicated that fragmentation does not begin to any appreciable extent until after 24hrs. Molecular weight standards were included in leftmost lane.

V. DISCUSSION

5.1 ANALYSIS OF APOPTOTIC CELL DEATH - QUALITATIVE AND QUANTITATIVE MEASURES

In vitro results from many laboratories over the last few years have suggested that apoptosis is an important cellular response to certain anticancer modalities. While *in vitro* models cannot completely duplicate the environmental conditions which exist in a tumor *in situ,* they do allow us to address mechanistic questions regarding cell death *in vivo.* A significant factor in this understanding is the apoptotic assay that is chosen. We have chosen four assays, each measuring a unique apoptotic characteristic, either morphological or biochemical. A comparison of the results shows a significant difference between MO59J and the other three cell lines. While M059J, the most radiosensitive cell line, was also the most sensitive to radiation induced apoptosis, the other radiosensitive cell line tested, M071 showed little apoptosis.

A comparison of the results also indicates that different assays provide different quantitative estimates of apoptosis. This adds to the difficulty in studying the process. The rate of apoptosis and the endonucleolytic pattern of DNA fragmentation is known to differ among cells lines, environments, time frames and induction agents. Modeling of the classic "apoptotic pathway" is extremely complicated because factors can inter-relate differently in different cells. Each assay allows one to take a "snapshot" of what is happening in that cell line at a precise moment in a specific environment. Understanding apoptosis as a physiological process, we must also understand it as a dynamic

process.

The most accurate measures of apoptosis would require multiple determinations not only within one assay, but with multiple assays. Each assay encompases inherent advantages and disadvantages. The decision as to which assay to use should involve the particular information being sought and the technical restrictions involved. While similar trends can be seen with each assay, for example M059J was consistently more suseptible to apoptosis than the other three cell lines, each provided a quantitatively different estimate of the percent apoptotic cells.

We have therefore concluded that because diverse endonucleolytic patterns can occur between different cell lines, it is necessary to record both morphological features with microscopy and the DNA degradation pattern with gel electrophoresis. In addition, we would suggest that other assays be used for more precise quantitative and temporal data, in order to better characterize the apoptotic susceptibility of a cell line. For clinical applications, methods based on electrophoresis may not be suitable due to time restrictions. In contrast, flow cytometry is a fast and efficient assay which gives clear, quantitative results from a small number of cells which can be obtained from a biopsy. DNA content analysis may be combined with TDT end labelling procedures to improve the sensitivity of the assay.

Other important apoptotic factors include those directly involved in the characteristic DNA fragmentation. Our studies of the apoptotic endonucleolytic process in glioma cells have revealed fragmentation characteristics corresponding to the first three levels of chromatin organization. ZIFE analysis of our glioma cell lines has shown a peak fragmentation size at approximately

300 kbp. These fragments are thought to arise from the release of 6 loops (or a rosette) of chromatin as they become detatched from the nuclear scaffold. The lack of smaller fragmentation to 180 bp nucleosome fragments would suggest that there is a low level of the necessary endonuclease or a topographical difference in chromatin structure such that the internucleosomal cleavage sites are inaccessible. In either case, the internucleosomal fragmentation does not appear to be an essential step of the apoptotic process occurring in these cells. The reorganization of the chromatin under the nuclear envelope may be the most important characteristic event, occurring in all cells prior to any DNA cleavage.

5.2 INTER- AND INTRA-TUMORAL HETEROGENEITY: DIFFERENCES IN EXPRESSION OF APOPTOSIS

The apoptotic suseptibility differences found among these cell lines may reflect the original tumor response, although this cannot be ascertained retrospectively. Tumors are complex cell populations in which cell loss and cell gain are occurrring simultaneously resulting in a "spectrum pattern of death". For example Arends *et al.* [71] suggest that the rate of cell turnover in tumors is determined by an intrinsic suseptibility to apoptosis and this characteristic differs between tumor cells. Not all tumor cells are capable of an apoptotic response even after relatively high doses of irradiation[65] and there is heterogeneity within the tumors that do show a response. We have confirmed these findings in human malignant glioma cell lines which show a response that differs between

cell lines (M059, M071 and M016) as well as among cells derived from the same tumor (M059J and M059K).

Several factors may contribute to this inter- and intratumor heterogeneity. Differentiation seems to be important for apoptotic susceptibility[69]. There may be residual hierarchical organization within our tumor cell subpopulations which reflects to some extent the normal tissue origins of the tumor. High grade gliomas are generally referred to as histologically pleomorphic in that they are composed of not one glial cell type but a mixture of several neoplastic glial elements[72]. In addition, the genetic expression of the cells is continually altered as a result of genetic instability and the influence of changing environmental factors. Cytogenetic studies have shown that tumors from different patients show different chromosomal complements (ploidy) despite showing similar histology. The heterogeneic response of our glioma cell lines in response to apoptotic induction is consistent with the genotypic and phenotypic heterogeneity which is characteristic of gliomas and suggests reasons why human malignant gliomas represent a form of cancer that continues to defy aggressive multimodality treatment. Our results have shown that three of four high grade gliomas studied are not sensitive to apoptosis induction. This would suggest that apoptosis is not a major mode of cell death in malignant gliomas. However a more extensive survey or other gliomas would be required to confirm this.

5.3 ARE THE FACTORS INVOLVED IN APOPTOSIS AND RADIATION SENSITIVITY SIMILAR?

Previous studies in murine models have suggested that tumors which show a high level of spontaneous apoptosis will also show an acute apoptotic response after radiation treatment. If this correlation were to hold for human tumors, apoptotic assays could have predictive value with regard to tumor response to radiotherapy. However, this predictive ability would be complicated by tumor heterogeneity, and the response of cells obtained from biopsies may not be indicative of how the tumor as a whole will respond.

Our glioma studies suggest an extension of this hypothesis. In three of four glioma cell lines there was a correlation between the spontaneous levels of apoptosis and the extent of radiation induced apoptosis. The background levels of apoptosis in our glioma cells were low and did not vary considerably (see Table II), indicating all have the capacity to initiate the apoptotic death program. However, since our test system is restricted to tumor cell lines, we cannot comment on whether or not these cells grown as solid tumors *in situ* would have had similar levels of spontaneous apoptosis.

Overall the percentage of M059J cells that die from apoptosis is much greater than that observed in the other three cell lines. There are two possible explanations for this. In the first instance, the process of apoptosis in cells showing extreme radiation sensitivity may be triggered in a manner similar to that found in more radioresistant cells. However, the effector mechanisms may differ. Alternatively, M059J cells may be predisposed to initiate the process

because they are unable to pass the DNA lesion sensoring checkpoint prior to mitosis. We know from previous studies[73] that there is a defect in the ability of M059J cells to repair double strand breaks. Sensing that its DNA is no longer intact, and unable to repair the damage, the cell may trigger apoptosis rather than wait to die after mitotic failiure. Although M071 has been characterized as another radiosensitive cell line, preliminary experiments indicate it is not DNA double strand break repair deficient (J. Turner, unpublished data).

Heterogeneity in response would suggest that the induction mechanism within cells differs after irradiation. The complexity of the pathways involved cannot be underestimated. In addition to the mechanistic differences between cell lines with respect to the apoptotic process, there may be many differences between the lines due to their malignant phenotypes. Therefore investigating the apoptotic mechanisms used by tumor cells may be inappropriate to understanding the mechanisms used by a normal cell, and vice versa.

The signalling response to radiation injury has generally been thought to involve several proteins including p53. In our glioma cells there is a lack of correlation between *p53* status, DNA-PK status, and apoptotic suseptibility (see table 1). This would argue that the *p53* pathway for DNA damage recognition and apoptosis initiation cannot be the only option available for these cells. This would also suggest that there are other DNA damage recognition proteins yet to be found - perhaps signalling through a radiation induced *p53* independent pathway. The fact that M071 does not induce apoptosis to the same extent as M059J would suggest that apoptosis is not contributing considerably to its radiosensitivity. Since the DNA-PK status of these cells was found to be in the normal range, it would suggest that a different

mutation may be responsible for the inherent radiosensitivity and that this radiosensitivity is independent of the cell's ability to undergo apoptosis.

5.4 EMT6 CELLS AND PDT APOPTOTIC INDUCTION.

The most striking and immediate cellular *in vitro* response observed following PDT in EMT6 cells is damage to membranes, particularly the plasma membrane. Within hours of treatment visible damage is characterised by formation of multiple membrane blebs, often very large, indicating severe membrane damage, after which cell lysis follows. Other membranes in addition to the plasma membrane may be at risk, including those of the nucleus, mitochondria, lysosome, golgi and endoplasmic reticulum. The damage to the membranes has generally thought to be due to oxidative stress caused when the illumination of the laser light source produces free radicals within the cell.

Although the apoptotic signal is commonly referred to as *nuclear*, there is also the possiblity that a signal can originate at the cytoplasmic membrane. Confocal microscopy studies[63] have shown the photosensitizer drugs to be localized primarily in the cytoplasmic and membrane regions of the cell. Subsequent damage to these membranes after laser illumination could result in an apoptotic signal being sent. Therefore, despite the fact that apoptosis ultimately results in the nuclear event of chromatin dismantling, the signal to begin the process may be initiated within the cytoplasmic regions of the cell. Photodynamic therapy in EMT6 cells may provide a model for future investigation of this phenomenon. In addition, membrane signalling could be involved in a secondary response to DNA damage, or reverse transduction from

the nucleus.

Regardless of how the signal is sent, EMT6 cells appear to have the cellular machinery necessary to undergo apoptosis. Results of these experiments indicate >30% of EMT-6 cells underwent apoptosis after PDT treatment suggesting that this may be a very important mechansim of cell death in these cells. The other interesting aspect of the PDT results is that the induction of apoptosis required both the presence of the photosensitizing agent and exposure to laser illumination. This may provide the opportunity for a greater selective treatment of tumor cells using fiber optics with little toxicity to the surrounding normal cells. The two photosensitizing drugs HBEA-R1 and HBBA-R2 both derived from the parent compound hypocrellin B have intrinsic differences in their chemical structure, however these differences do not seem to influence their respective abilities to induce apoptosis.

The results of the ZIFE with these cells after apoptosis induction were similar to those obtained with the glioma cell lines and suggest that there are many cell types that do not cleave their DNA to internucleosome fragments characteristic of DNA ladders. In addition to our glioma cells, EMT6 cells could serve as a model for determining which endonucleases are involved in the initial cleavage process.

If we hypothesize that many of the same factors that control the propensity of cultured cells to undergo apoptosis may also be responsible for regulating apoptosis *in vivo,* we may be able to use this *in vitro* system to investigate the molecular and biochemical mechanisms that regulate radiation and PDT induced apoptosis. Tumors are known to contain subpopulations which differ in their suseptibility to initiate this form of cell death. The regulatory

factors present in the environment have not yet been identified, but if we were able to manipulate this process, it may lead to an *in vivo* protection of normal tissues or an enhancement of apoptosis in tumors for therapeutic gain.

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4.2 PDT INDUCED APOPTOSIS

4.2.1 Fluorescence Microscopy.

Control, dark treated and light treated cells were examined at 4, 24 and 48 hours after treatment for evidence of apoptotic nuclear condensation, using the same methods described for radiation induced apoptosis. Table V shows the mean ± standard deviation percentage of apoptotic cells in each sample. Photomicrographs of apoptotic EMT6 cells are shown in figure 8.

Table VI

PDT Fluorescence Microscopy - Percent Apoptotic Cells per Slide

TREATMENT	4 hours	24 hours	48 hours
HBBA-R2			
control	2.5 ± 0.7	2.6 ± 0.9	3.4 ± 1.1
dark	3.6 ± 1.3	3.5 ± 1.2	4.9 ± 2.1
light	6.8 ± 0.7	11.6 ± 2.5	30.2 ± 3.5
HBEA-R1			
control	1.4 ± 0.5	n.d.	1.8 ± 0.3
dark	1.6 ± 0.9	2.0 ± 0.7	2.8 ± 0.6
light	2.8 ± 2.2	11.6 ± 2.6	41.7 ± 4.0

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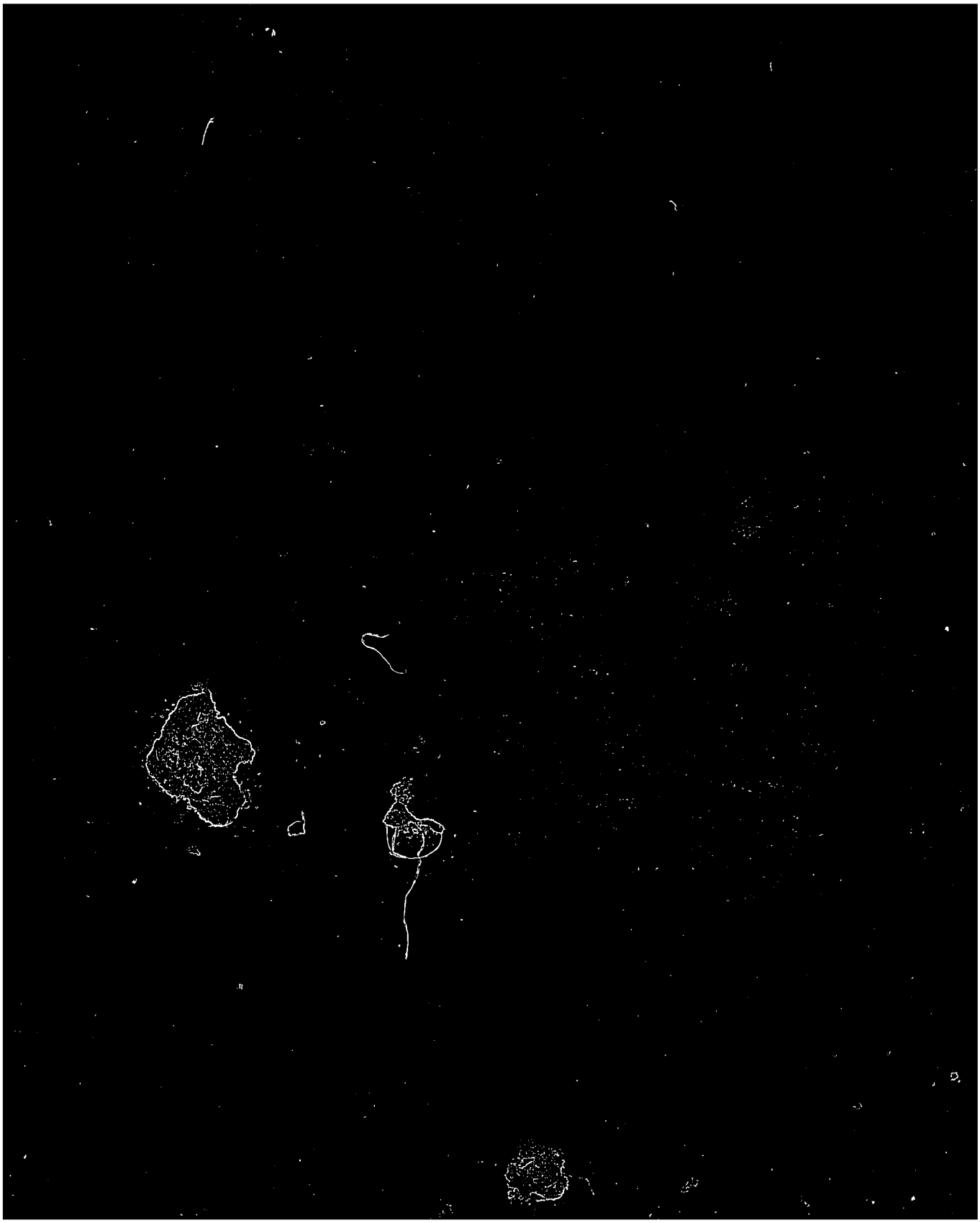


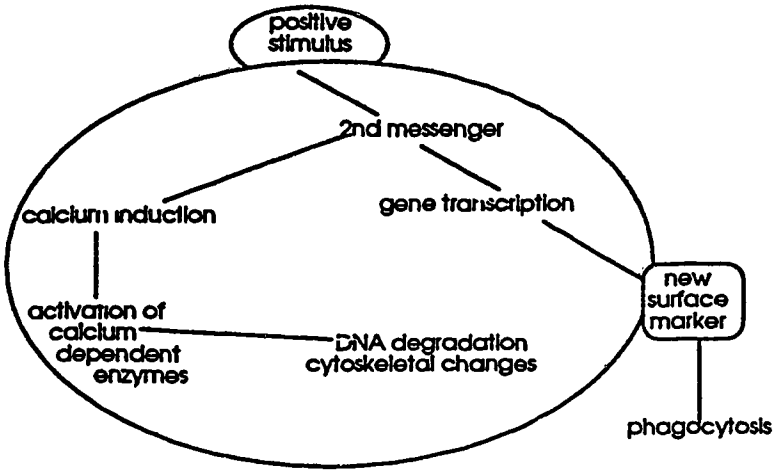
minimization of phenotype variation and elimination of genetic alteration. This mechanism can not only account for aberrant cell deletion but the deleted cells may be indirectly responsible for the survival of the other normal cells.

If apoptosis is indeed a protective mechanism for maintaining genetic fidelity it would require endogenous cellular factors capable of regulation. As with all aspects of physiology there is a compelling association with molecular events in the cell. A continuous stream of signals from outside the cell to its interior and exchange of signals between the nucleus and the cytoplasm are extremely important in order for the cell to be able to manipulate its genomic and metabolic processes according to external conditions. With an understanding of this *signalling net* it may be possible to understand the regulation of apoptosis in order to manipulate the process for therapeutic benefit.

1.2 APOPTOSIS AND CARCINOGENESIS

Cancer can be considered to be a disturbance in the growth control mechanism of the cell. However, a cell's ability to use apoptosis as a regulatory mechanism may be even more important than its proliferative ability. Some cancer cells divide more slowly than do some normal cells, nonetheless, the neoplasm still expands because of a prolonged life span in the cancer cells.

A cell can respond to injury (for example, radiation or chemotherapeutic drugs) in several ways: 1) it can delay cell division until the damage is repaired, 2) it can undergo apoptosis or 3) it can progress without interuption through the cell growth cycle. It has been suggested that cells that have become malignantly transformed have aquired the ability to bypass the first two



However, many discrepancies have arisen when researchers have tried to manipulate the proposed mechanisms in this model. Some of these complexities and their possible interpretations are discussed below.

1.5.2 Cohen' s Multi Mechanism Model

Cohen *et al.*[42] argue against a universal requirement for de novo expression of proposed *death genes* and offer the following model for the discrepancies seen between different cell types:

1. Much of the evidence for protein and RNA synthesis requirement comes from rodent thymocytes. These cells may be exceptions to the norm.

2. In some instances, protein and RNA inhibitors have been shown to block apoptosis, however, the possibility that this response was due to the effects of the inhibitors on other cellular processes has not been

have been shown to induce apoptosis in murine tumors[62] . The use of the current clinically approved photosensitizers available in the clinic has caused some concern for the toxic effects on normal tissue. For example, patients treated with hematoporphyrin derivatives can develop symptoms such as erythema, edema and pain in light exposed areas. Cutaneous photosensitivity arising from hematoporphyrins can remain for several months but is usually 6 - 8 weeks in duration[65]. New sensitizers are now being developed with a view toward an enhanced photodynamic effect, utilization of simpler light sources and elimination of concurrent skin photosensitization[63].

Hypocrellins A (HA) and B (HB) are two main pigments isolated from the parasitic fungi *Hypocrella bambusae Sacc* and *Shirai bambusicola P.* that are found in China. These pigments have a long history as traditional medicinal agents[62]. Two derivatives of the parent compound Hypocrellin B being developed at the University of Alberta/Cross Cancer Institute for use in photodynamic therapy include HBEA-R1 and HBBA-R2[67].

Table II

Fluorescence Microscopy - Percent Apoptotic Cells per Slide

Cell line	Control	24hrs after 10 Gy	48hrs after 10 Gy
M016	10 ± 3%	11 ± 3%	20 ± 7%
M071	9 ± 4%	9 ± 2%	15 ± 2%
M059J	12 ± 4%	19 ± 2%	22 ± 4% **
M059K	15 ± 4%	13± 3%	18 ± 4%

* These measurement represent mean ± standard deviation based on three slides per cell line.

**p<.035.

Figure 7

Photomicrographs of EMT6 Cells Stained with Propidium Iodide After Photodynamic Therapy

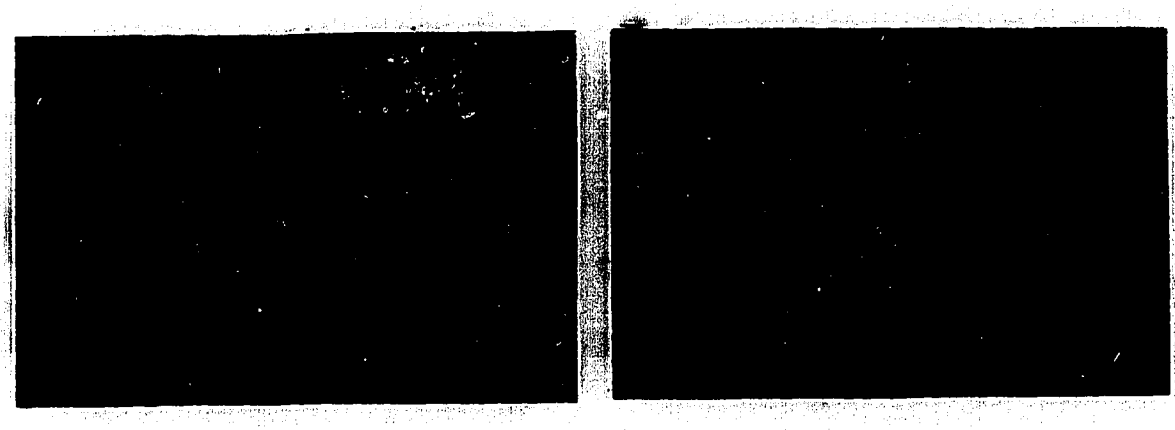


FIG 7. Typical patterns of condensed chromatin and apoptotic bodies found in EMT6 cells 24 hours after PDT treatment. Cells were stained with propidium iodide. 500X magnification is shown.

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