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# Apoptotic Events in Glioma Activate Metalloproteinases and Enhance Invasiveness

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Additional information is available at the end of the chapter

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## 1. Introduction

A cancer of the brain that arises from supporting tissue (glial cells) is referred to as a glioma. Glioblastomas are fast growing gliomas and can be referred to as a Grade IV astrocytoma or glioblastoma multiformes (GBMs). GBMs that affect the brain do not readily spread to parts of the body outside of the brain, but they are highly invasive and invade surrounding tissue in the brain. The persistent invasiveness, which is extremely difficult to reverse, is what makes GBMs so deadly. Therapeutic regimens for GBM and other high grade gliomas have fallen short of providing effective treatment. Clinical studies comparing chemotherapeutic agents have indicated increased tumor shrinkage and a very slight increase in median survival times, but no evidence for an increase in survival rates [1, 2]. More recent clinical studies employing surgery with the use of chemotherapeutic regimens in combination with radiation therapy or by receptor mediated growth hormone deprivation are slightly more effective in increasing median survival times, but again have had limited success in increasing the overall survival rate in patients being treated for primary or recurrent GBM [3-7]. Clinicians and neurosurgeons are diligently pursuing new treatments and provide hope for patients and their families [8, 9]. However, the difficulties of complete resection, the resistance to radiation and other therapies and particularly the intractable malignant invasiveness still remains at the root of the very poor survival prognosis for patients with GBM and other high grade gliomas. Despite extensive studies regarding the clinical aspects of GBM very little is known about the behavior of GBM during apoptosis. The ideas pursued in this paper are relevant to apoptotic cells within a glioma enhancing the invasiveness of the apoptotic resistant cells and thus contributing to the tumor's malignant properties.

Apoptosis is a type of cell death in which there is a multipath sequence of programmed events within the cell. These events require energy and lead to a suicidal self-destruction of the cell

without releasing intracellular components to the surrounding area. The apoptotic process is accompanied by numerous biochemical changes within the cell that are manifested by morphological changes which include blebbing, cell shrinkage, chromatin condensation and DNA fragmentation. The etymological origins of the word apoptosis is a Greek term meaning “falling off” and first appeared in a publication in 1972 by Kerr and his colleagues [10]. Over the past four decades apoptosis has been extensively studied [11]. Apoptosis is considered an essential physiological process in eukaryotes which impacts embryonic development, tissue homeostasis, wound healing, elimination of cells by the immune response and is an important process in the elimination of malignant or potentially malignant cells. For a tumor mass to arise, the cells within the cancerous tumor in some way had to escape apoptosis. The cancerous cell type that emerges requires clinical treatment by surgical, radiological and chemotherapeutic techniques. It is well known that all radiological and pharmacological treatments of cancerous cells are treatments that induce cell death by apoptosis. In fact, other than surgery, malignant cell types require a clinical treatment that induces cell death by apoptosis because the cellular debris and inflammation produced by massive necrosis of an extensive tumor would be systemically injurious to the patient. Thus, capable investigations of some of the mechanisms associated with apoptotic cell death in Glioblastomas can of themselves be significant. Beyond that however, the fact that Glioblastomas are usually highly invasive, but in general do not metastasize is what renders studies about invasiveness particularly relevant to clinical treatments of Glioblastomas [12]. In light of this, any contribution to furthering the understanding about the invasiveness of Glioblastomas would be significant and is likely to be well received.

Metalloproteinases are a group of enzymes that can break down proteins such as collagen and other extracellular matrix proteins. Because these enzymes need zinc or calcium to work properly, they are called metalloproteinases. It has been known for some time that metalloproteinases play an important role in the degradation of the extracellular matrix (ECM) [13, 14]. However, the basic action of metalloproteinases, which is the cleavage of proteins, has proven sufficiently sophisticated to orchestrate various functions in addition to degradation of the ECM [15, 16]. The metalloproteinase family includes: matrix metalloproteinases (MMPs) [17, 18], membrane type matrix metalloproteinases (MT-MMPs) [19, 20], a disintegrin and metalloproteinases (ADAMs) [21] and a disintegrin and metalloproteinases with thrombospondin type 1 motif (ADAMTs) [22]. The MMPs and the ADAMTs are secreted while the activated ADAMs and MT-MMPs remain anchored to the cell. All four of the metalloproteinase families have the ability to act on ECM proteins and growth factor receptors as substrates. The metalloproteinases have the capacity to regulate tumorigenesis [23], angiogenesis [24, 25], tumor growth [26], and invasiveness [16]. Metalloproteinases are naturally inhibited by specific tissue inhibitors of metalloproteinases (TIMPs) [27]. Thus, over the last two decades, it has become clear that metalloproteinases do more than degrade structural ECM proteins. Metalloproteinases are produced in an inactive zymogen form. One of the aims of this present report is to focus on the role of apoptosis with regard to the secretion and activation of metalloproteinases. Although metalloproteinases have been extensively studied, the total relevance of secreted MMPs or MT-MMPs with regard to invasiveness and malignancy of Glioblastomas is not completely resolved at present. It might be anticipated that metallopro-

teinases have important and yet to be discovered roles with regard to the invasiveness of Glioblastomas.

Anoikis is a form of cell death induced by inadequate or inappropriate cell–matrix interactions whereupon there is a loss of cell adhesion signals [28]. In the case of noncancerous cells, as they lose contact with the extracellular matrix an anoikic cell death response is initiated. Anoikic cell death proceeds via apoptotic programs and can be considered a natural protection against cells being detached and subsequently migrating from their primary site and becoming established outside of their normal environment. With regard to cancerous cells many of them are anoikic resistant. Anoikis is regulated by a variety of signaling pathways depending on the cell type [29]. The circumventing of anoikic cell death by cancer cells enables the cancer cells to migrate from their primary site and enter new environments and survive [30, 31]. Despite the fact that in general Glioblastomas do not metastasize, there is motivation to know more about the secretion and activity of metalloproteinases in anoikic resistant glioma cells. A focus of this present report is to address the question of whether apoptotic and anoikic resistant LN18 cells are an important factor with regard to metalloproteinase secretion and activation of the zymogen form. The question is significant given the nonhomogeneous state of a tumor which most likely consists of a mixture of apoptotic and non-apoptotic cells. As cells within a tumor become apoptotic micro cavities will be formed and not all of the cells will receive a full complement of cell adhesion signals. Thus, whether treated by radiation, chemotherapeutics or natural defenses, a tumor is most likely to exist as a milieu of dying apoptotic cells, anoikic resistant cells, and non-apoptotic cells that are resistant to treatment. The question of whether MMPs are activated or secreted from apoptotic or anoikic resistant cells is relevant to the possibility that loosely attached cells or therapeutically induced apoptotic cells can interact with apoptotic resistant cells and thus contribute to angiogenesis, tumor growth and the invasiveness of Glioblastomas. Experiments within this report have been done with the view that anoikis and the escape from anoikis are worthy of investigation in the context of MMP secretion; particularly in the context of the effects of activated MMPs upon GBM invasiveness.

## 2. Overall purpose

This report will first provide evidence that the apoptotic state of LN18 glioblastoma cells can be readily delineated and as such provides a convenient *in vitro* model to study effects of apoptotic cells upon the malignant properties of gliomas. In the context of metalloproteinase activation, an objective is to point out that in late apoptosis LN18 cells manifest a degradation of surface determinants including: growth factor receptors EGFR and IGF1-R, histocompatibility markers such as HLA-ABC, and a variety of cluster determinants including alpha and beta integrins. The degradation of surface determinants like integrins and growth factor receptors could be viewed as a full commitment to apoptosis through a decrease in survival signals. However, the perspective presented here is that Glioblastomas that lose growth factor signals and cell adhesion signals, even if dying, still have the capacity to affect the malignancy of those GBM cells within the tumor that are in a non-apoptotic state. In addition, it has been

presumed that GBM cells going into apoptosis is a condition that is totally beneficial to the patient. However, an aim of this report is to point out that there may be a down side to apoptosis that is worthy of consideration. In addition, anoikic resistance is thought of mostly in terms of cells traveling through the circulatory system and surviving to be the root cause of metastases [32]. Yet, anoikic resistant cells that lose their adhesion signals *in situ* may be supportive of the deadly invasive characteristics of the malignant non-apoptotic cells within the GBM tumor. More specifically the explorations of this report are intended to:

- present evidence for the presence and increased activation of metalloproteinases in LN18 glioblastoma cells as they proceed through apoptosis,
- address the possibility that MMP secretion increases in LN18 cells that are devoid of cell adhesion signals and,
- call attention to the idea that within the milieu of a glioblastoma, apoptosis may have a role in enhancing the invasiveness of the non-apoptotic cells.

### 3. Methods

#### 3.1. Cell culture conditions

The LN18 cell line (ATTC CRL-2610) was established in 1976 from a patient with a high grade right temporal lobe glioma. The cells are poorly differentiated, are adherent, and grow well in culture [33]. For cell maintenance, the cells were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub>, 95% air, and 100% humidity. Cells were maintained in 75 cm<sup>2</sup> flasks in Dulbecco's Modified Eagle Medium free of phenol red and supplemented with the dipeptide L-alanyl-L-glutamine (2mM), non-essential amino acids, pyruvate (100µg/ml), penicillin (100 units/ml), streptomycin (100µg/ml), amphotericin B (0.25µg/ml), HEPES (25mM), and fetal bovine serum (10%). Media and all tissue culture reagents were purchased from Invitrogen. Cells were plated from a trypsin digest (0.25% trypsin, EDTA). All plastic ware used for tissue culture and other experimentation including flasks, 96, 24 and 6 well plates were purchased from Fisher (Costar). Numerical data represent 3 to 4 replicates per sample. When establishing significant differences between samples ( $\alpha=0.05$ ) the data were analyzed by One Way Anova/Tukey Tests.

#### 3.2. Apoptotic inducing agents

The apoptotic inducing agents used in the report are MK886 and staurosporine. MK886 is a molecule reported to inhibit both five lipoxigenase activating protein (FLAP) [39] and peroxisome proliferator activated receptor- $\alpha$  (PPAR- $\alpha$ ) [34]. Staurosporine has been reported to inhibit protein kinase c [35]. Both MK886 and staurosporine are proven apoptotic inducing agents [36-40]. MK886 and staurosporine were purchased from Sigma. Apoptosis was induced by micro molar levels of inducing agent solubilized in DMSO and diluted to the appropriate concentration in the Dulbecco's reaction medium

### 3.3. Antibodies and reagents

Mouse monoclonal primary antibodies used for flow cytometry included: mouse anti-KLH (Biolegend), mouse anti- $\beta$  actin, mouse anti-integrins and mouse anti-HLA-ABC (Upstate, Chemicon, Linco) and goat anti-mouse-IgG conjugated to phycoerythrin (Jackson Laboratories).

## 4. Assays for apoptosis for LN18 cells in a monolayer

Classical apoptotic morphology was used as a manifestation of apoptosis. Early apoptosis was determined by phosphatidylserine (PS) translocation to the outer membrane as measured by flow cytometry manifested by annexin binding in the absence of propidium iodide uptake (Roche, Annexin-V-FLOUS kit) or annexin binding in the absence of uptake of 7-aminoactinomycin D (Molecular Probes).

Apoptosis was also detected by measuring the degradation and loss of activity of Poly(ADP-ribose) polymerase (PARP). The functional activity of PARP was determined using a PARP Activity Assay kit (R&D Systems). PARP activity extracted from cells and activated was measured by determining the level of incorporation of biotin labeled NAD into histones that are coated onto a microtiter plate. The color development was measured with a computer-assisted MR4000 Dynatech Microplate Reader at 450nm.

Late apoptosis was measured by the release of nucleosome histone complexes from the nucleus into the cytosol. The Cell Death ELISA kit (Roche) was used. The method includes a microtiter plate which has its wells streptavidin coated. The primary immunoreagent is a biotin labeled anti-histone antibody which binds to the streptavidin coated plate and histones within the experimental sample. The secondary antibody is a peroxidase conjugated anti-DNA that was added after sample addition. The assay detects soluble cytoplasmic DNA-histone complexes resulting from apoptotic nucleosomes that are tightly bound with core histones that appear in the cytosol before the plasma membrane disintegrates. The ELISA color development was measured with a computer-assisted MR4000 Dynatech Microplate Reader at 405nm.

Late apoptosis was also measured by the electrophoretic manifestation of DNA degradation. DNA extraction for electrophoresis was done according to the procedure described by Gong et al. [41]. Essentially, the cells were prefixed in 70% ethanol and the DNA was extracted with 0.2 M phosphate-citrate buffer at pH 7.8. The extract was sequentially treated with RNase A and proteinase K and then subjected to electrophoresis. After 12 hours of treatment with MK886 the DNA was extracted from  $3 \times 10^6$  LN18 cells and loaded in a 20 $\mu$ L volume into a 4% agarose gel. The gel was run at 85volts for 1h. The gels were subsequently stained with 0.4 $\mu$ g/ml of ethidium bromide and photographed under UV exposure in an Alpha Innotech Fluorchem 8800 photo imager.

Measuring the degree of nuclear condensation and fragmentation by staining with the DNA binding fluorescent dye 4',6-diamidino-2-phenylindole (DAPI) was another indicator of apoptosis that was used.

#### 4.1. Flow cytometry to detect surface proteins and apoptosis

The surface proteins of apoptotic cells were compared to the surface proteins of non-apoptotic cells. Following treatment the apoptotic and non-apoptotic cells were suspended, centrifuged and washed. The cells were then suspended in the appropriate buffer and reacted with the primary antibodies (mouse @ ITGA2, mouse @ ITGB2 or mouse @ HLA-ABC). Following incubation with the primary antibody the cells were rewashed and incubated with goat @ mouse IgG conjugated to phycoerythrin. The cells were then washed and assayed by flow cytometry. To measure apoptosis the dyes annexinV conjugated to fluorescein isothiocyanate (annexin -FITC) and 7-Amino-actinomycinD (7-AAD) were used. Dyes were added in the appropriate buffer. Following labeling, cells were analyzed with Becton-Dickinson FACSCalibur (San Jose, CA) using CellQuest Pro software.

#### 4.2. Real time RT-PCR

The RNA was isolated from cells using TRIZOL Reagent (Life Technologies). Reverse transcriptase generated c-DNAs were obtained using random hexamers with the high capacity archive kit from Applied Biosystems. C-DNAs were allowed to form for 2h at 37°C. Negative controls were generated by omitting the reverse transcriptase in the cDNA generating step. For the PCR step, the primers and Taq-Man fluorescent probes were purchased from Applied Biosystems. The primers were designed to span an intron to avoid amplification of any contaminating DNA. Real time PCR was performed using the Applied Biosystems Gene Amp 5700 system with the Taq-Man Universal PCR Master Mix. Relative mRNA levels were measured using the threshold cycle (Ct). The threshold cycle was defined as the cycle number that first gives detection of the PCR amplicon above a fixed threshold baseline set within the log phase of the plot of fluorescence versus cycle number. The amplicons were generated over 40 cycles where each cycle consisted of a 15 sec dissociation step at 92°C and a polymerization step at 60°C for 1 min. The  $\Delta$ Ct was calculated by subtracting the Ct value for the housekeeping gene  $\beta$ -actin (ACTB) from the Ct value for the gene of interest for the same c-DNA sample. The  $\Delta$ Ct of the vehicle (DMSO) control cells was then compared to the  $\Delta$ Ct of the MK886 treated cells.

#### 4.3. ELISA assay of matrix metalloproteinase proteins released from LN18 cells

The level of each MMP protein released into the supernatant from non-adherent anoikic LN18 cells incubated in polyhema coated flasks was measured by a sandwich ELISA (R&D Systems). Monoclonal antibodies specific for the human MMP were immobilized onto the surface of the wells of a plastic 96 well microtiter plate. 100 $\mu$ l of undiluted samples were added and following incubation (2h @ RT), the unbound material was washed away (4X) and a biotinylated goat anti-human MMP conjugated to horseradish peroxidase (HRP) was added and incubated for 2h @ RT. The cells were then washed 4X. The substrate tetra-methyl-benzidine (TMB) was added and allowed to develop for 30min. Sulfuric acid was added as a stopping solution. A BMG Labtech FLUOstar OPTIMA plate reader at 450nm was used to quantify the absorbance that resulted from the developed color of the substrate.

#### 4.4. Fluorometric assay of metalloproteinase activity

The enzymatic activity of released MMPs from suspended LN18 cells was tested using the Fluorescence Resonance Energy Transfer (FRET) peptide QXL520 -Arg-Pro-Leu-Ala-Leu-Trp-Arg-Lys(5-FAM)-NH<sub>2</sub> (ANASPEC). The hydrolysis of the FRET peptide by the pure recombinant MMP3 was used as a control. The fluorescence was monitored in microtiter plates using a FLUOStar OPTIMA Fluorometer at an Ex/Em=490nm/520nm. The complete activation of the released MMPs was insured by the use of 1mM of 4-aminophenylmercuric acetate (APMA).

#### 4.5. Matrigel measure of LN18 cell invasiveness

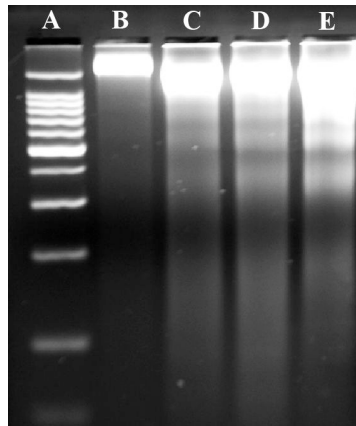
The invasiveness was measured *in vitro* using a BD Falcon matrigel matrix system. This system consists of a 24-multiwell plate where each well has an insert consisting of an 8.0 $\mu$  pore size polyethylene terephthalate (PET) membrane that has been uniformly coated with a layer of matrigel which serves as a reconstituted matrix *in vitro*. The coating process occludes the pores of the PET membrane and thereby blocks the non-invasive cells from migrating through the membrane. In contrast, invasive cells are able to degrade the matrigel coating of the membrane and migrate through the 8.0 $\mu$  pores of the PET membrane. The invasive non-apoptotic LN18 cells were stained with the lipophilic fluorescent dye DiI (Molecular Probes). Since the PET membrane effectively blocks the passage of light from 490-700nm at >99% efficiency only those stained cells that pass through the PET membrane are detected. Fluorescently-labeled cells that have not passed through the matrigel and the PET membrane are not detected by the bottom reading fluorescent plate reader. The chemoattractant in the lower chambers of the 24 well plate (below the matrigel insert) was DMEM medium that is 0.5% fetal bovine serum (FBS). Invasiveness was monitored using a FLUOStar OPTIMA plate reading fluorometer at an Ex/Em=549nm/565nm for 2h at 37°C.

## 5. Results

### 5.1. Characterization of apoptosis induced by MK886 in a monolayer of LN18 cells

DNA fragmentation is a classic indicator of apoptosis. Figure 1 is a photographed UV exposure of DNA agarose gels. The DNA was extracted from control LN18 cells and MK886 treated LN18 cells while in a monolayer. Following electrophoresis of the extracted DNA the gels were stained with ethidium bromide. Column A is the electrophoretic pattern of a 100bp standard. The following columns are the electrophoretic patterns for the medium control (column B), 10 $\mu$ M of MK886 for 12h (column C), 25 $\mu$ M of MK886 for 12h (column D) and 50 $\mu$ M of MK886 for 12h (column E). DNA fragmentation is a very late event in the apoptotic process. Not all cell types respond the same in the fragmentation of DNA. Some cell types give a very clear laddering and others produce more of a smearing of DNA similar to that found in states of necrosis. For apoptotic processes the nuclear DNA would have been previously condensed during the earlier stages of apoptosis distinguishing it from necrosis. The asynchrony and slowness of apoptotic en-

try can also contribute to the formation of wider smeared bands of degraded DNA. We have found that the more compact a monolayer of LN18 cells is the slower is the apoptotic entry as indicated by the slower development of morphological changes of rounding up and blebbing of the cells. The electrophoretic patterns of Figure 1 show that there is more degradation of DNA when LN18 cells are treated with increasing concentrations of MK886.



**Figure 1.** Electrophoretic patterns of DNA fragmentation of apoptotic LN18 cells. Column A: 100 bp standard. Column B: non-apoptotic control. Columns C-E: increasing concentrations of apoptotic MK886 inducing agent of 10, 25 & 50  $\mu\text{M}$  respectively for 12h.

Another classical indicator of apoptosis is the degradation and loss of enzymatic activity of Poly(ADP-ribose) polymerase (PARP). PARP is a family of multifunctional enzymes the most dominant of which are PARP-1 and PARP-2. One of the major roles of PARP-1 is the post-translational modification of nuclear proteins induced by DNA strand-breaks that contributes to the survival of injured proliferating cells [42]. The catalytic activity of PARP is immediately stimulated by DNA strand-breaks [43]. As delineated in the Methods, activated PARP-1 cleaves NAD into nicotinamide and ADP-ribose and catalyzes the transfer of ADP-ribose units from NAD<sup>+</sup> to target nuclear proteins. Apoptosis initiates a caspases degradation of PARP which is manifested by a decrease in PARP's enzymatic activity [44]. The data of Table 1 show the activity of PARP for control cells held in medium and MK886 treated cells at 10, 25 and 50 $\mu\text{M}$  of MK886. The decrease in activity of PARP is indicated by the decrease in color development as demonstrated by the decrease in absorbance at 450nm. In addition to the measures of apoptosis shown here, MK886 induced apoptosis in monolayer LN18 cells has been shown to be caspases dependent and is exhibited by the release of nucleosomes, annexinV binding to phosphatidylserine in the presence and absence of nuclear staining, changes in morphology and changes in the fluorescent intensity of mitotracker deep red indicating changes in mitochondrial oxidative function [45]. The alteration of the mitochondrial function implies that apoptosis is induced in the LN18 cells via a mitochondrial pathway.



Treatment	A <sub>450 nm</sub>
Medium	0.756 +/- 0.136
Medium W/O bio-NAD	0.155 +/- 0.058
10 μM MK886	0.481 +/- 0.173
25 μM MK886	0.308 +/- 0.079
50 μM MK886	0.296 +/- 0.094
¹LN18 cells were treated for 10h at indicated MK886 concentrations while in a monolayer	

**Table 1.** MK886 Treatment of LN18 Cells Inhibits PARP Activity<sup>1</sup>

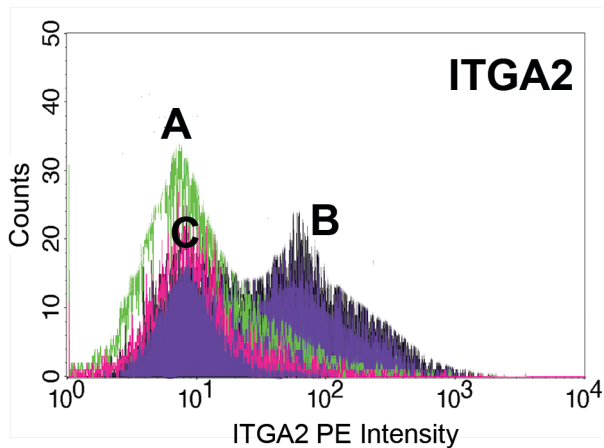
## 5.2. Degradation of surface determinants of monolayered LN18 cells in late apoptosis

The data presented here and the previous assessments of the time course and characteristics of apoptosis in monolayered LN18 cells render the system useful for further studies about the role of apoptosis and anoikis in enhancing GBN malignancy. LN18 cells losing contact with the extracellular matrix as they proceed through apoptosis is of interest. Integrins are a class of molecules relevant to cell adhesion to extracellular matrix proteins. Integrins exist as heterodimers consisting of linked alpha and beta chains forming a family of 24 distinct integrins. Integrins were first recognized as a family of receptors in a seminal paper put forth by Hynes in 1987 [46]. Since that time, it has become established that integrins have roles in addition to being receptors for cell adhesion proteins [47, 48]. Integrins act as membrane transduction receptors with the ability to promote a variety of signals to the cytoskeleton which activate many intracellular signaling pathways. An important property in the resistance to apoptosis is the ability of the beta1 integrin subunit (designated ITGB1 or Integrin β1) to bind extracellular domains and promote the formation of the Focal adhesion kinase-1 (FAK) complex. The FAK complex in turn results in activating the PI 3-kinase/Akt signaling pathway which promotes survival and delivers anti-apoptotic signals [49]. This raises the possibility that in the normal process of apoptosis there is a commitment by the apoptotic LN18 cells to degrade those surface determinants that promote survival signals and resistance to apoptosis.

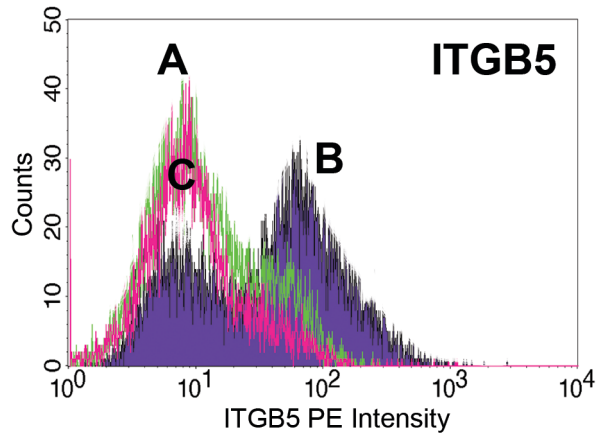
Figure 2 and Figure 3 are both composites of flow cytometry histograms (Curves A, B and C) showing phycoerythrin fluorescence intensity vs. cell counts. The curves were obtained by using mouse primary IgG antibodies specific for the integrin in question followed by goat anti-mouse-IgG conjugated to phycoerythrin. The intent of Figure 2 is to illustrate the density of the integrin subunit IGA2 (α2) on the surface of the LN18 cell as the cell progresses through apoptosis. The subunit α2 in combination with the subunit ITGB1 (β1) forms the dimer receptor that binds collagen which is a ubiquitous extracellular matrix protein [50, 51].

Similarly to Figure 2, Figure 3 illustrates the density of the integrin subunit ITGB5 (β) on the surface of the LN18 cell vs. cell count as the cell progresses through apoptosis. The integrin subunit β5 in combination with αV recognizes the short motif Arg-Gly-Asp (RGD motif) which

can be found in many proteins of the extracellular matrix [52]. Curves labeled A (green) of Figures 2 and 3 show the non-specific labeling that was obtained by using mouse anti-KLH as a primary antibody. Curves labeled B (purple) of Figures 2 and 3 indicate the fluorescence obtained by the mouse antibodies specific for each growth factor receptor for LN18 cells in mid apoptosis. For curves B, the cells were treated for 10h while in a monolayer with 50 $\mu$ M of MK886. The bimodal curve indicates that the population consists of cells that have high and low density of integrin determinants on the surface of the cells. Curves labeled C (red) indicate the fluorescence obtained by the mouse antibodies specific for each growth factor receptor for LN18 cells treated with MK886 for 15h. A clear down shift in the intensity of the growth factor histograms can be seen for the 15h MK886 treated cells (red) as compared to the cells treated for 10 h (purple). The down shift in integrin fluorescence intensities of the 15h MK886 treated cells fall almost completely into the region of non-specific binding indicating a high degree of degradation of the integrin determinants.



**Figure 2.** Flow cytometry histograms showing the density of integrin- $\alpha$  5 on the surface of LN18 cells in mid and late apoptosis. Curve A: @KLH non-binding control. Curve B: bimodal histogram of LN18 cells in mid apoptosis. Curve C: LN18 cells in late apoptosis.



**Figure 3.** Flow cytometry histograms showing the density of integrin- $\beta$  5 on the surface of LN18 cells in mid and late apoptosis. Curve A: @KLH non-binding control. Curve B: bimodal histogram of LN18 cells in mid apoptosis. Curve C: LN18 cells in late apoptosis.

### 5.3. RT-PCR of integrins

It is possible that the observed decrease in the density of the integrins on the surface of the LN18 cells is due to a decrease in expression rather than a degradation of the integrin determinants. To answer this we measured the expression of a number of integrins in control LN18 cells as compared to apoptotic LN18 cells. Table 2 shows the results obtained by real time reverse transcriptase polymerase chain reaction (real time RT-PCR) as a measure of message for specific integrins. The data were obtained from an amplification plot of the fluorescence signal versus cycle number. The numbers in the columns of Table 2 show the cycles to threshold (Ct values) for  $\beta$ -actin and the specific integrin subunits listed in column 1. The  $\beta$ -actin was used as a housekeeping control. The data of Table 2 show the Ct value for non-treated LN18 cells (2<sup>nd</sup> column) and cells exposed to 50 $\mu$ M MK886 for 10h (3<sup>rd</sup> column). The changes in Ct values ( $\Delta$ Ct) for each mRNA were obtained by subtracting the Ct value of the DMSO control cells from the Ct value for the MK886 treated cells at 10h and 15h. The Ct values for  $\beta$ -actin and the integrin subunits increased by about a cycle indicating a slight decrease of message as the LN18 cells proceeded through apoptosis. This slight increase in cycle number to threshold could not account for the large decreases in surface integrins shown in Figures 2 and 3. A normed ( $\Delta\Delta$ Ct) was calculated for each sample by subtracting the  $\Delta$ Ct value of  $\beta$ -actin from the  $\Delta$ Ct value for the gene of interest. The Ct values for the calculation of the 10h  $\Delta\Delta$ Ct values (4<sup>th</sup> column) are shown above, but the Ct values at 15h for the calculation of the 15h  $\Delta\Delta$ Ct values (5<sup>th</sup> column) are not shown.

Gene Designation	Ct Control 10h	Ct MK886 10h	$\Delta\Delta$ Ct MK886 10h	$\Delta\Delta$ Ct MK886 15h
$\beta$ - ACTIN (ACTB)	17	18	NA	NA
INTEGRIN- $\alpha$ 5 (ITGA5)	19	20	0	1
INTEGRIN- $\alpha$ V (ITGAV)	19	20	0	-1
INTEGRIN- $\beta$ 1 (ITGB1)	19	20	0	0
INTEGRIN- $\beta$ 3 (ITGB3)	23	24	0	0
INTEGRIN- $\beta$ 5 (ITGB5)	20	21	0	1

**Table 2.** Effect of MK886 on the level of specific mRNAs in LN18 cells

#### 5.4. Degradation of LN18 cell surface determinants is mediated by metalloproteinases

There is the question of whether the degradation of surface determinants due to apoptosis is specific or whether there is a gross loss of surface determinants as the cells progress through apoptosis. An additional question is, are there specific proteases that are activated that degrade the surface determinants of LN18 cells as they go into apoptosis? Human leukocyte antigens ABC (HLA-ABC) are within the major histocompatibility complex class I and are virtually ubiquitous in tissues. Thus, the HLA-ABC complex was chosen to determine if they also are degraded in apoptotic cells and if the degradation can be reversed by various protease inhibitors.

The flow cytometry output of Table 3 was obtained by using mouse primary IgG antibodies specific for HLA-ABC determinants followed by goat @mouse IgG conjugated to phycoerythrin. The first column of Table 3 is a list of protease inhibitors. The numerical values within the columns of Table 3 represent the integrated fluorescence intensity of the phycoerythrin dye where a decrease in integrated intensity indicates a decrease in the density of HLA-ABC determinants on the surface of the LN18 cell. The data of Table 3 show that as LN18 cells progress through apoptosis, induced by 50 $\mu$ M of MK886, there is a decrease in the surface determinants of HLA-ABC as compared to vehicle control cells (2<sup>nd</sup> column vs. 3<sup>rd</sup> column). For the non-apoptotic controls (2<sup>nd</sup> column) the proteolytic inhibitors were added at 7h after the initiation of the incubation. For the apoptotic MK886 treated cells (3<sup>rd</sup> column) no proteolytic inhibitors were added. For the cells that had both MK886 and proteolytic inhibitor added, the proteolytic inhibitor was added 7h after the MK886 was added (4<sup>th</sup> column). The cells were allowed to incubate for an additional 6h after the addition of the protease inhibitors. It can be seen from the third column of Table 3 as compared to the second column that treatment with the matrix metalloproteinase inhibitors GM6001 and MM-2 MM-9 demonstrated a significant inhibition of the apoptotic loss of HLA-ABC determinants. Some of the proteasome inhibitors severely exacerbated the LN18 cell's loss of determinants while the calpain inhibitors had little to no effect. Similar effects were obtained for the cell surface receptors EGFR, INGF1-R and INGF2-R (not shown). Serine protease inhibitors such as aprotinin and soybean trypsin inhibitor partially reversed surface determinant degradation induced by apoptosis indicating

that degradation of surface determinants is the result of a combination or interplay of serine proteases and metalloproteinases (not shown). The reversal of the degradation of cluster determinants by metalloproteinase inhibitors is relevant to the studies showing matrix metalloproteinase secretion from LN18 glioblastoma cells in suspension.

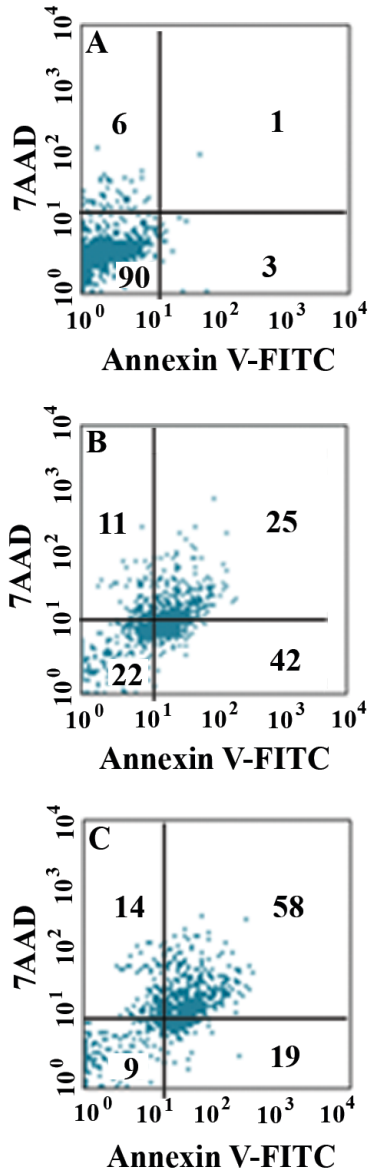
Type of Proteolytic Inhibitor	Control No MK886	MK886 50µM	50µM MK886 Plus Inhibitor
ZVAD-FMK (50µM)	3299	2001	2335
CALPAIN III (50µM)	3449	2465	2350
CALPEPTIN (50µM)	2690	1647	1425
MG-132 (50µM)	2858	1791	TOO LOW
PSI (50µM)	2880	2089	1003
GM6001 (10µM)	2907	1914	2854
MM-2 MM-9 (50µM)	3069	1722	2759
PD 150606 (50µM)	3822	2153	742
ALLN, MG101 (50µM)	3534	1795	211
ZVAD-FMK (50µM) Broad caspases inhibitor			
CALPAIN III (50µM) Inhibits calpain 1&2			
CALPEPTIN (50µM) Inhibits calpain 1&2 and papain			
MG-132 (50µM) Proteasome inhibitor that also inhibits NF-κB			
PSI (50µM) Proteasome inhibitor that also inhibits NF-κB and chymotrypsin			
GM6001 (10µM) Broad matrix metalloproteinase inhibitor			
MM-2 MM-9 (50µM) Broad matrix metalloproteinase inhibitor			
PD 150606 (50µM) Calpain 1 inhibitor: directed against the calcium binding site			
ALLN, MG101 (50µM) Inhibits Calpain 1&2, cysteine proteases, proteasomes, IκB-α, IκB-β, ubiquitin-proteasome complexes, arrests cell cycle at G <sub>1</sub> /S			

**Table 3.** Effect of Proteolytic Inhibitors upon Apoptotic Decrease of the HLA-ABC Determinants on the Surface of LN18 Cells

### 5.5. Apoptosis induced by MK886 and staurosporine in LN18 cells held in suspension and devoid of integrin signaling

The panels of Figure 4 are flow cytometry outputs and are plots of annexin conjugated to fluorescein isothiocyanate (annexinV-FITC) versus 7-amino-actinomycinD (7AAD) staining of the LN18 cells. The flow cytometry data of annexinV-FITC versus 7AAD is a classical test for apoptosis. The data of Figure 4 are a measure of the degree of apoptosis in LN18 glioblastoma cells while suspended in polyhema coated flasks. Panel A of Figure 4 illustrates vehicle control cells which were iced while in suspension and then immediately stained. Pharmacologically induced apoptosis of the suspended cells was brought about by treating the cells with 50 $\mu$ M of MK886 for 8h (panel B) and for 10h (panel C). Numbers denoted in quadrants of each panel represent the percentage of cells in each quadrant. Viable cells that are not positive for annexinV-FITC or 7AAD are neither apoptotic nor necrotic and are represented in the lower left quadrant. Necrotic cells devoid of apoptosis that stained positive for 7AAD, but not for annexinV-FITC, are represented in the upper left quadrant. Apoptotic cells devoid of necrosis that stained for annexinV-FITC, but not 7AAD, are in the lower right quadrant and late apoptotic/necrotic cells that stained for both annexinV-FITC and 7AAD are represented in the upper right quadrant. Panel A of Figure 4 shows that 90% of control cells are devoid of annexinV-FITC or 7AAD staining indicating no initial necrosis or apoptosis. LN18 cells in suspension devoid of extracellular signaling rapidly go into apoptosis. It can be seen that 67% of the cells are stained positive for annexinV-FITC by 6h (sum of the 2 right quadrants of panel B) due to treatment with MK886. When the LN18 cells are in suspension devoid of integrin signaling, they proceed into apoptosis much more rapidly than when in a monolayer. After 8h of stimulation with MK886, 77% of the cells stain positive with annexinV-FITC (sum of the 2 right quadrants of panel C). The data of Figure 4 are relevant to those experiments which show that LN18 cells rendered apoptotic while in suspension enhance the invasiveness of non-apoptotic LN18 cells.

Nucleosomes are essentially DNA-histone complexes which in non-apoptotic cells are confined to the nucleus. An additional well established test for apoptosis is the release of nucleosomes from the nucleus into the cytosol. To further demonstrate apoptosis for cells that lack integrin stimulation, LN18 cells were treated with a variety of concentrations of staurosporine while in suspension in polyhema coated flasks. The data of Table 4 show the results of assaying for the release of DNA-histone complexes from the nucleus into the cytosol for LN18 cells in polyhema coated flasks. The LN18 cells were treated with staurosporine at the concentrations indicated for 8h. The cells were then centrifuged and washed and then treated with lysis buffer. The supernatants of the lysed cells were then transferred to a streptavidin coated 96 well microtiter plates and tested for DNA-histone complexes by ELISA using anti-histone-biotin-antibody followed by peroxidase conjugated to anti-DNA. The development of peroxidase substrate was measured by reading a Dynatech Microplate Reader at 405nm. It can be seen that 1 $\mu$ M of staurosporine can induce the release of a significant amount of histone-associated DNA-fragments into the cytosol by 8h. Furthermore, the release of DNA fragments was inhibited by the broad caspases inhibitor ZVAD-FMK, indicating that staurosporine-induced degradation of DNA in LN18 cells is caspases dependent.



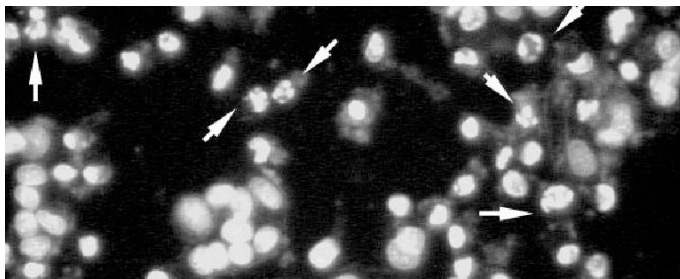
**Figure 4.** Flow cytometry data showing nuclear staining vs. translocated phosphatidylserine ( PS) staining as LN18 cells proceed through MK886 induced apoptosis while in suspension and devoid of integrin stimulation.

Staurosporine Concentration	A <sub>405 nm</sub>	SD
Medium Control	0.145	+/-0.051
0.5 $\mu$ M	0.297	+/-0.081
1.0 $\mu$ M	0.643	+/-0.073
2.0 $\mu$ M	0.862	+/-0.136
2.0 $\mu$ M + 50 $\mu$ M ZVAD-FMK	0.197	+/-0.026

<sup>1</sup>LN18 cells were treated for 8h at indicated staurosporine concentrations.

**Table 4.** Apoptotic Release of Nucleosome from LN18 Cells Treated with Staurosporine while Suspended in Polyhema Coated Flasks<sup>1</sup>

The LN18 cells shown in the micrograph labeled Figure 5 were incubated in suspension with 1 $\mu$ M staurosporine for 6h. After the incubation the cells were harvested by centrifugation and then fixed in 70% ethanol. The cells were then centrifuged and washed and suspended in 2ml of phosphate buffered saline. For the assay 10 $\mu$ l of stock (0.1mg/ml) 4,6-diamidino-2-phenylindole (DAPI) was added to the 2ml PBS. After completion of the staining with DAPI, the cells were observed on a slide with an inverted microscope set for DAPI. The arrows of the micrograph labeled Figure 5 show nuclear condensation within LN18 cells. Nuclear condensation is another established indicator of apoptosis. The condensation correlates with the nucleosome release and is clear indication that both staurosporine and MK886 induce late apoptotic effects in LN18 cells that are not integrin stimulated as early as 8-10h.

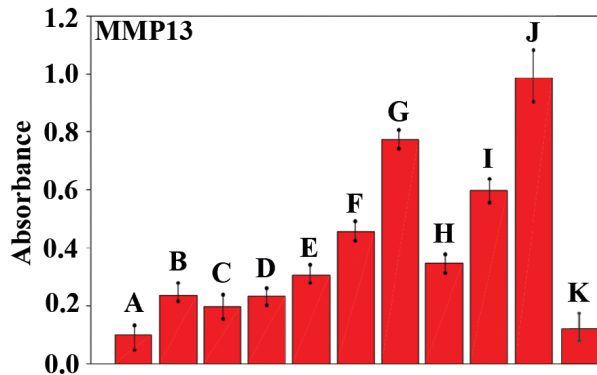


**Figure 5.** The arrows within the micrograph show nuclear condensation within LN18 cells in suspension upon the induction of apoptosis by treating the cells with 1 $\mu$ M Staurosporine for 6h.

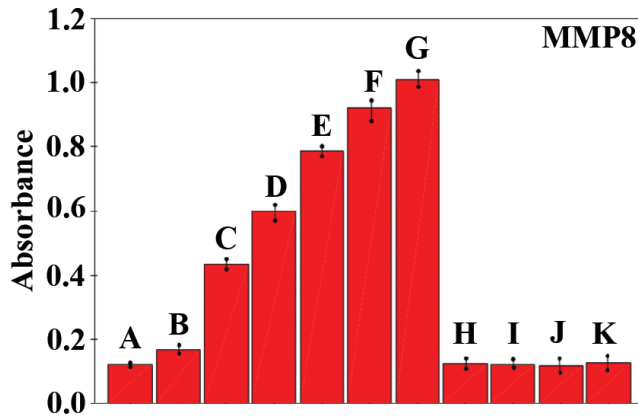


### 5.6. LN18 cells that are devoid of integrin stimulation release matrix metalloproteinases

Cells held in suspension in polyhema coated flasks are devoid of those extracellular matrix transduction signals that are derived from cells being stimulated by extracellular matrix proteins. The data of Figures 6 and 7 were accumulated by an ELISA technique as presented in the Methods. The data show the release of MMP13 (Figure 6) and the lack of release of MMP8 (Figure 7). The MMPs were released in the absence of pharmacological stimulation (MK886 or staurosporine) by LN18 glioblastoma cells when there is merely a loss of cell adhesion signals brought about by incubating the cells in suspension in polyhema coated flasks. The samples for the Figures 6 and 7 are as follows: Bar A is the medium control; Bar B is the supernatant of the ice control where the cells were suspended, but not allowed to progress at 37°C; Bars C through G illustrate dose responses for pure MMP13 (Figure 6) and pure MMP8 (Figure 7) which are 0.5ng/ml (Bar C), 1.0ng/ml (Bar D), 2.0ng/ml (Bar E), 4.0ng/ml (Bar F), and 6.0ng/ml (Bar G). Bars H through J are the supernatants of LN18 cells suspended in polyhema coated flasks at  $1.5 \times 10^6$  cells/ml in Dulbecco's medium with 2% FBS for 2hrs (Bar H), 4hrs (Bar I), 6hrs (Bar J) at 37°C. Bar K is the response from LN18 cells held for 6hrs in suspension, but in the presence of  $1.78 \times 10^{-4}$  M cycloheximide. Other metalloproteinases released from LN18 cells while being held in suspension include MMP1, MMP2 and MMP3 (not shown).



**Figure 6.** ELISA measurement of released MMP13 from LN18 cells in suspension. Bars A&B are controls. Bars C & D are the dose responses for pure MMP13. Bars H-J are measures of the MMP13 released from cells held in suspension for 2, 4 & 6h respectively. Bar K is the cycloheximide control at 6h.



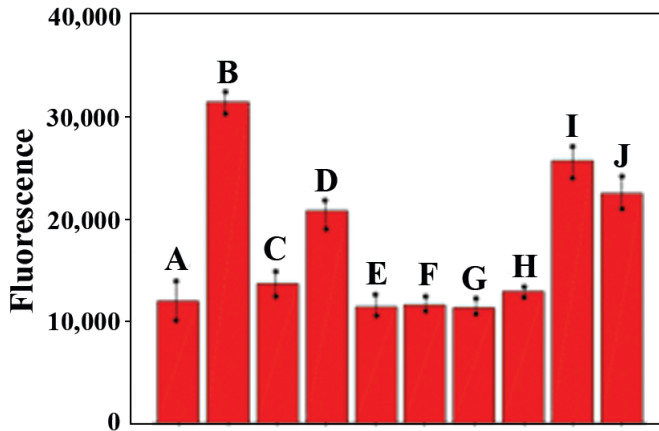
**Figure 7.** ELISA measurement of released MMP8 from LN18 cells in suspension. Bars A&B are controls. Bars C&D are the dose responses for pure MMP8. Bars H-J are measures of the MMP8 released from cells held in suspension for 2, 4 & 6h respectively. Bar K is the cycloheximide control at 6h.

As determined by RT-PCR there was a slight increase in message (one to two cycles) for the metalloproteinases shown in Figures 6 and 7 when the cells were held in suspension for 6h at 37°C (not shown). Examining Bars H-J of Figure 6 as compared to cells not allowed to progress at 37°C in suspension (ice control Bar B) it can be seen that there is a significant amount of MMP13 release from the LN18 cells held in suspension. Whether the zymogen form of the MMPs is stored in the cytosol was not determined but it appears from Bar K that, even if much of the metalloproteinase are stored in the cytosol prior to secretion, translation is essential indicating that the production of some type of protein is required for secretion to proceed. Figure 7 shows a lack of secretion of MMP8. In addition to MMP8, MMP7 and MMP9 were not secreted in amounts that were detectable (not shown).

### 5.7. Metalloproteinase inhibitors block the MMP activity released from LN18 cells held in suspension

The data of Figure 8 illustrate the inhibition of the total metalloproteinase activity released into the supernatant from LN18 cells held in suspension. The cells were incubated in polyhema coated flasks in the presence of DMEM supplemented with 1% FBS and growth hormones for 6hrs. As described in the Methods, the MMP activity in the supernatant of the suspended cells was measured fluorometrically using the broad MMP substrate QXL520 -Arg-Pro-Leu-Ala-Leu-Trp-Arg-Lys(5-FAM)-NH<sub>2</sub>. The total secreted metalloproteinase activity included a variety of MMPs secreted from the LN18 cells including that of MMP-1, -2, -3 (not shown) in addition to MMP13 (shown in Figure 6). Bar A is the metalloproteinase activity of the supernatant of cells left on ice (control blank). Bar B is supernatant of the 6hr incubation positive control showing the secreted enzymatic activity tested in the absence of inhibitors. For the remaining samples, the supernatant of the 6hr suspended cells containing the secreted

metalloproteinases was tested for enzymatic activity in the presence of the following inhibitors: 10mM EDTA (Bar C); 5mM EDTA (Bar D); 5mM of the zinc chelator 1,10 phenanthroline (Bar E); 2.5mM of 1,10 phenanthroline (Bar F); 50 $\mu$ M of the broad metalloproteinase inhibitor GM6001 (Bar G); and, 25 $\mu$ M of GM6001 (Bar H). Bar I shows the effect of the solvent isopropyl alcohol (12.5%), which was used to dissolve the 1,10 phenanthroline. Bar J shows the effect of the solvent DMSO (12.5%), which was used to dissolve the GM6001. The data indicate that metalloproteinases secreted from the suspended LN18 cells have enzymatic activity that was inhibited by a variety of known MMP inhibitors.



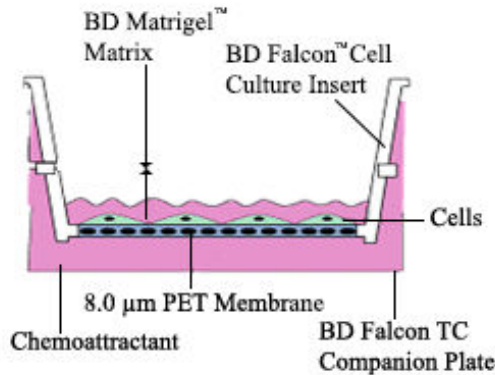
**Figure 8.** Inhibition of metalloproteinase activity secreted from LN18 cells. Bar A is the non-releasing ice control. Bar B is the non-inhibited sample. Bars C-H show reduced activity by a variety of known metalloproteinase inhibitors. Bars I & J are solvent controls.

### 5.8. Effect of apoptotic LN18 cells upon the invasiveness of non-apoptotic LN18 cells

The invasiveness was measured *in vitro* using a BD Falcon matrigel matrix system. This system consists of a 24-multiwell plate where each well has an insert consisting of an 8.0 $\mu$  pore size polyethylene terephthalate (PET) membrane that has been uniformly coated with a layer of matrigel which serves as a reconstituted matrix *in vitro*. The coating procedure occludes the pores of the PET membrane. Those cells capable of degrading the matrigel coating of the PET membrane will migrate through the 8.0 $\mu$  pores of the PET membrane. For clarification, a diagram of one well of the 24 well plate matrigel system is shown in Figure 9.

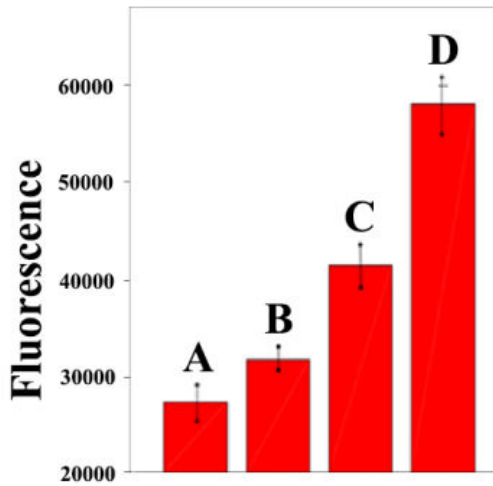
The system requires a chemoattractant in the lower chamber. Constituents within fetal bovine serum (FBS) are known to act as chemoattractants, which enables the cells to migrate from the serum free upper chamber toward the lower chamber. Various concentrations of FBS were tested and it was determined that 0.5% FBS in Dulbecco's medium was an effective concentration (not shown). The invasive cells are stained with the lipophilic fluorescent dye DiI (Molecular Probes). Since the PET membrane effectively blocks the passage of light from

490-700nm at >99% efficiency, only those stained cells that pass through the PET membrane were detected. Fluorescently-labeled cells that have not passed through the matrigel and the PET membrane were not detected by the bottom reading fluorescent plate reader. Invasiveness was monitored using a FLUOStar OPTIMA plate reading fluorometer at an Ex/Em=549nm/565nm for 2h at 37°C. It was determined that  $2 \times 10^5$  stained LN18 cells in the upper chamber that were capable of migrating into the lower chamber gave a strong fluorometric signal.



**Figure 9.** Diagram of one well of the 24 well plate matrigel system.

The intent of using the matrigel invasive system was to measure the effect various numbers of apoptotic LN18 cells have upon the invasiveness of a fixed number of stained non-apoptotic LN18 cells. Figure 10 shows the effect of combining in the upper chamber a variety of apoptotic LN18 cells upon  $2 \times 10^5$  of the invasive, non-apoptotic, DiI stained LN18 cells. Before mixing the two cell populations apoptosis was induced separately from the non-apoptotic cell population. The apoptotic population was obtained by stimulating the LN18 cells with  $1 \mu\text{M}$  of staurosporine for 8h. The apoptotic LN18 cells were then washed but not stained. The non-apoptotic cells were stained with DiI and the mixtures of the non-apoptotic and apoptotic cells were aliquoted into the upper chambers in Dulbecco's medium supplemented with growth factors and 0.5% bovine serum albumin (BSA) but devoid of FBS. Dulbecco's medium supplemented with 0.5% FBS was the chemoattractant within the lower chamber. Samples A through D represent the following cell mixtures where in each case only the  $2 \times 10^5$  non-apoptotic cell populations were stained with DiI. Sample A is  $1.7 \times 10^5$  unstained apoptotic LN18 cells +  $2 \times 10^5$  non-apoptotic DiI stained LN18 cells with no FBS in the lower chamber (no chemoattractant control). Sample B is  $1.7 \times 10^5$  unstained non-apoptotic LN18 cells +  $2 \times 10^5$  non-apoptotic DiI stained LN18 cells (non-apoptotic equal cell count control). Sample C is  $2.7 \times 10^5$  unstained apoptotic LN18 cells +  $2 \times 10^5$  non-apoptotic DiI stained LN18 cells. Sample D is  $4.7 \times 10^5$  unstained apoptotic LN18 cells +  $2 \times 10^5$  non-apoptotic DiI stained LN18 cells. Only sample A was devoid of FBS in the lower chamber. It can be seen from samples C and D of Figure 10 that increasing populations of apoptotic LN18 cells enhance the invasiveness of the of the non-apoptotic LN18 cells.



**Figure 10.** Enhanced invasiveness of non-apoptotic LN18 cells by the presences of apoptotic LN18 cells. Bar A no chemoattractant control. Bar B invasiveness of non-apoptotic cells in the absence of apoptotic cells. Bars C&D increased invasiveness of the non-apoptotic cells by increasing populations of apoptotic cells.

## 6. Conclusions

The main focus of this report was examining the effects of apoptotic glioblastoma cells upon the non-apoptotic glioblastoma cell population. Overall, the data show that the effects are in large part mediated by metalloproteinases.

A summary of the findings of this study are as follows:

- Apoptosis induced in LN18 cells, that are in a monolayer and integrin stimulated, is accompanied by an enhanced activation of metalloproteinases on the surface of the LN18 cells.
- Matrix metalloproteinases are secreted from LN18 cells that are in suspension and devoid of integrin signals, even in the absence of the pharmacological stimulation of apoptosis.
- The matrix metalloproteinase released from LN18 cells can be made to exhibit enzymatic activity which is reversed by metalloproteinase inhibitors.
- As LN18 glioblastoma cells progress through apoptosis, there is a degradation of surface determinants including, growth factor receptors, histocompatibility markers such as HLA-ABC, and a variety of cluster determinant including alpha and beta integrins. The degradation takes place in mid to late apoptosis. Metalloproteinase inhibitors blocked the degradation of surface determinants even when added after the cells where fully committed to apoptosis.

- By the use of Real-Time RT-PCR, it was determined that, overall, the down-regulation of surface proteins that takes place in late apoptosis is not mediated by an interruption of transcription mechanisms.
- Apoptotic LN18 glioblastoma cells when mixed with non-apoptotic LN18 cells enhance the invasiveness of the non-apoptotic LN18 cells

As aforementioned, when a malignancy is diagnosed and is subjected to primary treatment some of the cells will be more resistant to apoptosis than others which will inherently result in a mixture of apoptotic and viable non-apoptotic tumor cells. The existence of a mixture of apoptotic and non-apoptotic tumor cells is particularly true during recurrence and the required therapy that inevitably induces apoptosis which leads to a population of apoptotic cells within the tumor. As treatment progresses beyond the primary stage, mutated cells and resistant cells that escape apoptosis will proliferate. Thus, the sustained cell population of a tumor will consist of non-apoptotic cells capable of invading surrounding tissue while coexisting with a dying population of apoptotic cells. This view that recurrent GBM tumors consist of cells that are refractory to treatment while being highly invasive, and exist as a mixed population of apoptotic and non-apoptotic cells, was the stimulus for this study.

There have been elegant studies comparing non-cancerous glial cells to gliomas and demonstrating elevated membrane-type metalloproteinases in gliomas [53]. There are also studies demonstrating membrane-type metalloproteinases expression and function during glioma invasion [54, 55]. These studies have provided a footing and direction for this present study. The important idea here is that there are metalloproteinase activating processes that are taking place in apoptotic and anoikic cells. What is different about the approach proposed here is that we are not comparing glioma cells to normal glial cells, but rather investigating the presence of, and activity of, metalloproteinases in glioma cells as they progress through apoptosis.

It can be concluded from the data of this report that the membrane bound metalloproteinases in LN18 glioblastoma cells are surprisingly active during the late phases of pharmacologically induced apoptosis. In addition, matrix metalloproteinases are secreted from LN18 glioblastoma cells in early anoikis. One of the hallmarks for the induction of anoikis is the absence of cell adhesion signals. It can be assumed that the secreted matrix metalloproteinases, when activated, have effects on the pericellular matrix and adjacent cells. Even if the anoikic cells are apoptotic resistant, because metalloproteinases are secreted in early anoikis, apoptotic resistant cells devoid of adhesion signals could have the capacity to affect the surrounding matrix and nearby cells.

Most metalloproteinases are synthesized and secreted in a latent zymogen form. Furin-like proteases are known to act as sheddases acting on membrane type metalloproteinases [56]. Also there are type II transmembrane serine proteases (TTSPs), and possibly other proteases, present on the surface of glioma cells [57-59]. From the data presented here, it is reasonable to suspect that during the process of apoptosis there are secreted furins and possibly membrane-bound TTSPs that are capable of converting latent membrane bound or secreted metalloproteinases to their active form.

To reiterate, the conventional assumption is that ongoing apoptosis within a malignant tumor is desirable with no effects that may be harmful to the patient. What has been presented here

raises the possibility that within the mixed cell population of LN18 glioma cells and non-apoptotic LN18 cells, the apoptotic dying cells are capable of enhancing the invasiveness of the LN18 cells that are not apoptotic. As far as we know, investigating the invasive synergy between a mixture of apoptotic and non-apoptotic cells is somewhat neglected as an approach. The data of this study can be distinctive and be complementary to other studies directed toward the long standing problem of the malignant invasiveness of high grade gliomas.

## 7. Future studies

We look to identify which membrane type metalloproteinases (MT-MMPs and ADAMs) are on the surface of the LN18 glioblastoma cells and whether there is an increase in synthesis and their presence during apoptosis. One would surmise that inhibition of metalloproteinase activity would be an effective clinical treatment. There have been a good number of clinical trials for a variety of cancers determining the effectiveness of metalloproteinase inhibition upon patient improvement and survival rates [60-65] Unfortunately such studies have been disappointing particularly those clinical trials that have focused on glioblastomas [66,67]. The role of metalloproteinases with regard to invasiveness of GBM appears to be more complex than the ability of metalloproteinases to degrade the extracellular matrix (ECM). The experiments presented here raise questions about the role of furins, serine proteases, anoikis and apoptosis and their interrelationship to metalloproteinases and invasiveness. To expand upon the ideas presented here there is a need to determine whether the synthesis, secretion and presence of furins and TTSPs increase with apoptosis and whether these proteases are responsible for the activation of the latent zymogen form of metalloproteinases. Investigating the interplay between furins, TTSPs and the increased presence and activation of metalloproteinases during apoptosis in the context of the ability of apoptotic glioblastoma cells to enhance the invasiveness and motility of glioblastoma cells is a natural extension of what was presented here. Of particular interest is whether the apoptotic cells are acting on the extracellular matrix or there are direct effects upon the invading non-apoptotic cells. Findings utilizing the LN18 glioblastoma cell line will need to be tested in a variety of available glioma cell lines.

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