Chapter 4

Apoptotic Increases in Pericellular Protease Activity and Glioblastoma Escape from Immune Surveillance

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Abstract

This chapter presents evidence that the \textit{in vitro} enzymatic activities of serine proteases and metalloproteinases increase when apoptosis is induced in glioblastoma cells. What is distinctive about the approach of this report is that cancer cells are not compared to normal cells but rather there is an emphasis on the changes in glioblastoma cells as they progress through apoptosis as compared to non-apoptotic glioblastoma cells. The glioblastoma cell lines used were U118-GM, U87-GM and the LN18. Apoptosis was detected by morphological changes and flow cytometry measurements of changes in mitochondrial activity and violet fluorescent reactive dye [VFRD] staining. The data accumulated are presented in the context of pharmacologically stimulated apoptosis and the non-stimulated apoptosis, referred to as anoikus, brought about when cells are held in suspension devoid of cell/cell or extracellular matrix stimulation. The broad caspases inhibitor Z-VAD-FMK inhibited the processes of apoptosis and anoikus. Disintegrin and metalloproteinase cell surface antigens [ADAMs] and the serine proteases matriptase and furin were detected on the surface of the glioblastoma cells using protein specific primary antibodies followed by flow cytometry detectable phycoerythrin linked secondary antibodies. Increases in activity of metalloproteinases and serine proteases were detected in apoptotic and anoikic glioblastoma cells. The enzymatic activities of serine protease and metalloproteinase were measured by colorimet-
ric and fluorometric analysis using appropriate substrates. Apoptotic and anoikic induced metalloproteinase activity was inhibited by the broad metalloproteinase inhibitor GM6001. Soybean trypsin inhibitor was used to block the serine protease activity demonstrated by the anoikic and apoptotic glioblastomas. By ELISA and flow cytometry it was demonstrated that the process of apoptosis degraded the cell surface class I histocompatibility determinants HLA-ABC and the complement component C3a that was generated in solution. The data are presented in the view that glioblastoma tumors exist as a mixed population of apoptotic and non-apoptotic cells with the apoptotic cells being capable of degrading cell surface cluster determinants that are important stimulants of natural and acquired immune responses.

**Keywords**

Glioblastoma; Apoptosis; Cluster Determinants; Metalloproteinases; Serine Proteases; Invasion; Extracellular Matrix; Immune Suppression; Growth Factor Receptors; Integrins

**Introduction**

Therapeutic regimens for glioblastoma multiforme [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]. 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 glioblastoma [3-7]. The basis for treatments have been and are being pursued [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. It is well known that all radiological and pharmacological treatments of GBMs 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, which is the natural mechanism of cell death that avoids the cellular debris and inflammation produced by massive necrosis. The fact that GBMs are usually highly invasive, but in general do not metastasize [10], is what renders studies about the role of serine proteases and metalloproteinases particularly relevant to glioblastomas.

Apoptosis is a programed form of cell death that was first described by Kerr et. al. in 1972 [11]. Over the following decades the morphological and biochemical fea-
tures of apoptosis have been extensively investigated [12]. Spontaneous, non-pharmacological induced apoptosis under conditions devoid of cell/extracellular matrix interactions has been coined anoikis [13,14]. For a tumor mass to arise the cells within the tumor, in some way, escape apoptosis. Escape can come about through mechanisms mediated by the receptor tyrosine kinase family members or inherent defects in the cells’ apoptotic mechanisms [15] or resistance to the patient’s immune system [16-18]. Thus, when such a tumor mass is diagnosed, and is subjected to primary treatment, some of the cells will be more resistant to apoptosis than others. This 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. 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.

There have been studies demonstrating elevated matrix and membrane-type metalloproteinases in gliomas [19-24] and studies demonstrating increased metalloproteinases expression and function during glioma invasion [25-31]. An important component of malignant invasiveness is the degradation of the extracellular matrix [ECM]. It has been known for some time that metalloproteinases play an important role in the degradation of the ECM [32,33]. The basic action of metalloproteinases, which is the cleavage of proteins, has proven to orchestrate various functions with regard to degradation of the ECM [34,35]. Included in this process are metalloproteinase families, which are: matrix metalloproteinases (MMPs) [36,37], membrane type matrix metalloproteinases (MT-MMPs) [38,39], a disintegrin and metalloproteinases (ADAMs) [40] and a disintegrin and metalloproteinases with thrombospondin type 1 motif (ADAMTs) [41]. The MMPs and the ADAMTs are secreted while the activated ADAMs and MT-MMPs remain anchored to the cell. Consequently, ADAMs and MT-MMPs dependent proteolysis of the ECM and other substrates occurs at or near the cell membrane. All four of the metalloproteinase families have the ability to act on ECM proteins and growth factor receptors as substrates. Although the total relevance of metalloproteinases in glioblastoma function and malignancy is not completely clear at present, it is widely anticipated that MMPs, MT-MMPs and ADAMS have important and diverse roles. This chapter will demonstrate that ADAMs 9,10,12 and 17 are present on the surface of glioblastoma cells and that there is an increase in metalloproteinase activity as glioblastoma cells proceed through the process of apoptosis.

With regard to serine proteases, pericellular proteolysis in cancer cells was originally focused on the clas-
sic plasminogen–plasmin system [42-47]. This was then extended to membrane anchored serine proteases, which are ubiquitous in normal tissue and found to be overexpressed in several types of cancer with particular interest in matrix disruption and other effects of Type I and Type II Transmembrane Serine Proteases [48-56]. This chapter will present data that show matriptases are present on the surface of glioblastoma cells and that there is an increase in serine protease activity as glioblastoma cells proceed through the process of apoptosis.

There are questions regarding the interplay between Type I Transmembrane Serine Proteases such as matriptases and the activation of metalloproteinases. Furin is known to reside within the golgi apparatus and is in the family of proprotein convertases. With regard to its presence in cells, furin is virtually ubiquitous [57] and is a protein that is synthesized as a zymogen that can undergo intramolecular autocatalytic activation [58]. Furin can be activated within the golgi apparatus or can be transported out of the golgi apparatus and activated into its catalytically active form to act as a convertase at the plasma membrane or as a secreted convertase outside the cell. Following activation, furin is involved in converting numerous zymogen forms of enzymes [59 -62] including some forms of plasma membrane bound ADAMs [63-65]. This chapter illustrates that furin is present within glioblastoma cells.

It is becoming increasingly clear that gliomas can secrete the immunosuppressive cytokines particularly interleukin 10 [66-71]. Gliomas are a complex mixture of cells with microglia being the dominant resident cells that infiltrate the tumor [72]. Microglia are the key innate immune cells of the brain [73]. Although in healthy central nervous tissue microglia have a variety of phenotypes with different roles, they can be stimulated to develop into resident macrophages and perform immunological functions [74]. It has been reported that microglia have the capacity, directly or indirectly, to induce apoptosis in glioblastoma cells [75,76]. However, in their activated state microglia have been shown to enhance glioblastoma multiforme cell migration and invasion [77]. What is missing in the arguments regarding either invasiveness or natural and acquired immunity are the characteristics of apoptotic cells. Whether apoptosis is induced chemotherapeutically, radiologically, or by natural and acquired immunity, the role of apoptotic glioblastoma cells has been overlooked in the immune resistance and invasive properties of the glioma tumor [78,79]. This chapter is presented in the context that apoptotic glioblastoma cells have an increase in activity of metalloproteinases and serine proteases that can degrade cell surface molecules important to natural and acquired immune responses, while simultaneously having the capacity to degrade the extracellular matrix thereby contributing to the survival and invasive properties of gliomas.
Methods

Cell Types and Maintenance

The LN18 cell line (ATCC, CRL-2610) was established in 1976 from a patient with a right temporal lobe glioma [80]. The U87-MG (ATCC, HTB15) and U118-MG (ATCC, HTB14) cell lines were first isolated from patients afflicted with brain glioblastoma multiformes by Ponten and his colleagues in 1966 [81]. All three cell lines are considered Grade IV astrocytomas and are transplantable as xenographs into nude mice [80, 82, 83]. The cells are anchorage independent and grow well as monolayers in culture. Cells were maintained at 37°C in an atmosphere of 5% CO₂ at 100% humidity in 75cm² flasks in Advanced DMEM (F12) supplemented with the dipeptide L-alanyl-L-glutamine (2mM), non-essential amino acids, penicillin (100 units/ml), streptomycin (100µg/ml), amphotericin B (0.25µg/ml), HEPES (10 mM), and fetal bovine serum (5%). Cells were subcultured by trypsinization (0.25% trypsin, EDTA). All tissue culture reagents were obtained from Gibco.

Apoptotic Inducing Agents

The inducing agents used were staurosporine (STAURO, Sigma) and the lipoxygenase inhibitor MK886 (3-[[1-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropylindol-2-yl]-2, 2-dimethylpropanoic acid, Sigma). Staurosporine has been well studied [84] and, although not currently a clinically employed drug, it is relevant to current explorations regarding the efficacy of small-molecule kinase inhibitors in the treatment of solid cancers including glioblastoma [85]. Five lipoxygenase activating protein (FLAP) binds arachidonate and facilitates its interaction with 5-lipoxygenase (ALOX-5) [86, 87]. It is well established that MK886 inhibits the action of FLAP and blocks the formation of leukotrienes generated by the ALOX-5 pathway [88]. Inhibition of the lipoxygenase pathway has been shown to induce apoptosis in glioblastoma cancer cells [89]. STAURO and MK886 were dissolved in dimethyl sulfoxide (DMSO, Sigma) and diluted to the indicated concentrations in the culture medium.

Microscopic Examination of Cell Morphology

For microscopic examination cells were plated onto 8 chambered glass slides at 2×10⁴ cells/chamber. Following adherence and treatment the cells were fixed with 0.1% paraformaldehyde-PBS (Sigma) for 15min at room temperature and then permeabilized with 0.5% Triton X-PBS (Sigma) for 15min at room temperature. For actin and vinculin staining cells were incubated with Alexa Fluor phalloidin (Thermo Fisher) and anti-vinculin (Abcam) for 45min in the dark at room temperature. For actin and vinculin staining cells were incubated with Alexa Fluor phalloidin (Thermo Fisher) and anti-vinculin (Abcam) for 45min in the dark at room temperature. Following washing with PBS, cells were incubated with goat anti-mouse-IgG conjugated to Cy3 (Jackson Immuno Research) for 45min in the dark at room temperature. Following washing with PBS, cells were incubated with goat anti-mouse-IgG conjugated to Cy3 (Jackson Immuno Research). Cells were then washed with PBS and the monolayer was examined by fluorescent microscopy (Nikon Diaphot).
Data Accumulated by Fluorescence-Activated Cell Sorting Analysis

Flow cytometry data were accumulated using a two laser, 4 color Flow cytometry acquisition and Analysis instrument (Becton-Dickinson, San Jose CA) with Cell Quest Pro software or BD LSRFortessa (4 lasers) instrument using FACS Diva 8.0 software. A minimum of 10,000 events were collected for each sample. Instruments were located at the West Virginia University Flow Cytometry and Single Cell Core Facility.

Demonstrating Apoptosis by Dot Plots and Mitochondrial Function

Apoptosis was determined by changes in the mitochondrial mass and oxidative function indicated by changes in fluorescent intensity of the mitochondrial membrane binding dye MitoTracker Deep Red 633 (Thermo Fisher). Apoptosis was further assayed by changes in membrane permeability with Violet Fluorescent Reactive Dye (VFRD, Thermo Fisher) staining detected by the increase in intensity due to the increased amount of dye in the cytosol binding to the amino groups within the cytosol. Following labeling with either MitoTracker Deep Red or VFRD flow cytometry plots were acquired as described above.

Flow Cytometry Analysis of HLA-ABC Determinants on the Surface of Glioblastoma Cells

Apoptosis was induced in cells by MK886 at a concentration of 50μM to cells adhered as a monolayer in 96 well plates. The proteolytic inhibitors were added at 7h following the addition of MK886 to the monolayer. The plates were allowed to incubate for an additional 4h after the addition of the protease inhibitors. The cells were then harvested by the use of enzyme free cell dissociation buffer (Gibco). Mouse monoclonal and purified rabbit primary antibodies for HLA-ABC (Abcam) were used as a primary antibody. The secondary antibody was F(ab)_2 -goat anti-rabbit IgG conjugated to phycoerythrin. Flow cytometry data were then accumulated as described above.

Flow Cytometry Assay for ADAMs, Furin and Matriptase

The density of ADAMs, furin and matriptase on the surface of cells was measured by flow cytometry. Rabbit anti-ADAMs 9, 10,12 and17 (Abcam), furin and matriptase (R&D Systems) primary antibodies were all used at a concentration of 5μg/ml. F(ab)_2 -goat anti-rabbit IgG conjugated to phycoerythrin was used as the secondary antibody at a concentration of 2.5μg /ml. Rabbit anti-KLH (Sigma) was used as a primary antibody in the isotype-negative control. Flow cytometry data were then accumulated as described above.
Metalloproteinase and Serine Protease Activity by Fluorescence

The fluorogenic peptide substrate Boc-Arg-Val-Arg-Arg-AMC (Enzo Life Sciences) was used to detect the enzymatic activity of furin and matriptase types of serine proteases while MCA-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2 (Enzo Life Sciences) was used to detect the enzymatic activity of metalloproteinases. Serine protease inhibitor, soybean trypsin inhibitor, and metalloproteinase inhibitor (GM6001, Sigma) along with the apoptotic inhibitor Z-VAD-FMK (Sigma) were used. The serine protease activity was measured by the AMC fluorescence generated in 96 well plates with Ex 380 nm and Em 460 nm and the metalloproteinases activity measured by the MCA fluorescence with Ex 320 nm and Em 405 nm using a FLUOStar OPTIMA Fluorometer.

C3a Destruction by Apoptotic Cells as Measured by ELISA

The levels of C3a generated by zymosan (Sigma) acting on C3 in human serum (Valley Biomedical) were assayed by ELISA in apoptotic and non-apoptotic U87-MG cells. C3a was generated by zymosan (1x10^7 particles/well) and 20% normal human serum. Degradation of C3a was determined in the presence of apoptotic and non-apoptotic U87-MG cells (5x10^4 cells /well). Cells were in suspension in polyhema coated wells of a 96 well plate and reacted for 8h with 5µM Staurosporine. C3a levels in the supernatant were diluted 1/8,000 before assaying by ELISA. The 1/8000 dilution of the zymosan control was about 5ng/ml indicating that the undiluted reaction concentration was about 40µg/ml. C3a was assayed using a BD Opt EIA Human C3a ELISA Kit with the substrate 3,3',5,5'-Tetramethylbenzidine (TMB). Absorbance was read at 450 nm.

Results

Morphological Changes of Apoptotic Glioblastoma Cells

Figure 1 is a fluorescent micrograph (600X) of apoptotic morphological changes of a LN18 glioblastoma cell as the cell progresses through apoptosis. The actin cytoskeleton is visualized by phalloidin-488 (micrographs A, C, E) while vinculin is illustrated by mouse anti-vinculin followed by goat anti-mouse IgG conjugated to Cy3 (micrographs B, D, F). LN18 cells that were originally attached in a monolayer show actin stress fibers (micrograph A) and concentrations of vinculin within focal adhesions on the outer edge of the cell (micrograph B) when in early apoptosis. Morphological changes after being treated with 50μM of MK886 for 13h show rounding (micrographs C, D) and apoptotic blebbing by18h (micrographs E, F).

Figure 2 illustrates a non-stained micrograph (200x) of LN18 cells suspended in polyHema coated wells of a 24 well plate. Micrograph A illustrates spheroids of cells formed within 1h after plating. Micrograph B shows the separation of the spheroids and slight shrinkage of the
LN18 cells after being treated with 1µM of staurosporine for 3h.

**Figure 1:** Micrographs illustrating changes in morphology as LN18 glioblastoma progresses through apoptosis.

Micrographs A, C, E: Images created upon being stained with phallodin-488.

Micrographs B, D, E: Images created upon immune staining of vinculin with Cy3 conjugated antibodies.

Staurosporine is faster acting than MK886 as an apoptotic-inducing agent. Also, cells in suspension proceed through apoptosis much faster than cells that are adhered to an extracellular matrix. The micrographs of Figure 2 indicate that there are pericellular enzymes activated as the LN18 cells progress through apoptosis.

**Figure 2:** Separation of spheroid LN18 cells as they proceed through apoptosis.

Micrograph A: Image of spheroids of LN18 cells in early apoptosis.

Micrograph B: Image of individual LN18 cells of dissociated spheroids as cells progressed through apoptosis.

**Measures of Apoptosis**

Figure 3 illustrates changes in mitochondrial membrane function of adherent LN18 glioblastoma cells due to treatment with 1µM Staurosporine (STAURO) for 4h as compared to non-stimulated control cells. The cells were in a monolayer and adhered to the extracellular matrix. The histograms represent fluorescence intensity of Mi-
toTracker Deep Red 633 dye where a decrease in fluorescence indicates a decrease in mitochondrial membrane function, which is indicative of a mitochondrial mediated apoptotic pathway. The Mean Fluorescent Intensity (MFI) for the non-treated DMSO vehicle control LN18 Cells was approximately 1000 units. There was a significant down shift in MFI to a value of 100 units due to treatment with 1µM STAURO.

Figure 3: Mitochondrial function in apoptotic and non-apoptotic LN18 cells.

Figure 4 shows the dot plots for LN18 cells suspended in polyHEMA coated wells and labeled with AnnexinV-488 and Violet Fluorescent Reactive Dye (VFRD), and analyzed by flow cytometry as described in the Methods. The apoptotic-inducing agent was 1µM Staurosporine (STAURO). The initial non-treated DMSO vehicle control LN18 cells show 93% viable non-apoptotic cells (lower left quadrant). As manifested by the AnnexinV-488 vs VFRD dot plots, treatment of the suspended LN18 cells with 1µM STAURO for 4h shifted the population of LN18 cells to a mixture of mid-apoptosis shown in the upper right quadrant (18.8%) and late apoptosis shown in the upper left quadrant (77.9%).

Flow Cytometry Data for ADAMs and Serine Proteases on the Surface of LN18 Glioblastoma Cells

The cells for Figures 5 and 6 were non-apoptotic and grown attached as a monolayer and removed from the monolayer by treatment with a non-enzymatic cell dissociation buffer as described in the Methods. The panels of Figure 5 demonstrate flow cytometry data for 9,10,12 and
17ADAMs (a disintegrin and metalloproteinase) on the surface of U118-MG glioblastoma cells. The panels of Figure 6 illustrate the matriptase and furin proteins present on the U118-MG cells. The histograms show phycoerythrin fluorescence intensity vs cell count for the U118-MG cell surface antigens for the ADAMs and the serine proteases as designated. The primary antibodies were directed to the pro-domain of the ADAMs, the amino region of the furin and the catalytic region of matriptase molecules. The median intensity values for the ADAMs 9,10,12 and 17 are 50.48, 90.58, 93.06 and 66.42 respectively. The values are compared to a median intensity value of 27.76 for the anti-KLH isotype control. The histograms of Figure 5 with increased median intensity of the phycoerythrin fluorescence relative to the isotype controls indicate that ADAMs 9,10,2 and 17 are present on the surface of U118-MG cells. Likewise the median intensity values of Figure 6 for the furin and matriptase histograms are significantly higher than the KLH control indicating that the serine proteases are present on the U118-MG cells.

Figure 5: ADAMs 9,10,12 and 17 on the surface of U118-MG glioblastoma cells. The symbol @ in the panels indicates the word anti- of the primary antibodies. MI in the panels refers to Median Intensity.
Fluorescence Activity Illustrating Protease Activity on the Surface of LN18 Cell

The position put forth here is that when membrane bound ADAMs and serine proteases are activated they are capable of degrading cell surface determinants of cells within a glioma tumor or within the milieu of cells surrounding the tumor. The graphs of Figures 7 and 8 examine metalloproteinase and serine protease activity in LN18 glioblastoma cells as they progress through apoptosis and anoikis. In both Figures 7 and 8 enzyme specific fluorescent substrates were used.

**Figure 6:** Serine proteases furin and matriptase on the surface of U118-MG glioblastoma cells. Anti-KLH (keyhole limpet hemocyanin) panel is the isotype control.

**Figure 7:** Graphs showing LN18 metalloproteinase activity as the cells proceed through apoptosis and anoikis. Relative fluorescence generated by action of cells upon MCA-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2, a metalloproteinase fluorescent substrate.
Apoptosis was induced by 1μM STAUNO while the cells were in suspension. The upper and lower panels of Figure 7 show that LN18 cells treated with 1μM STAUNO (blue curve A) and undergoing anoikis (green curve C) exhibit increasing metalloproteinase activity. The upper panel of Figure 7 shows that the serine protease inhibitor, soybean trypsin inhibitor at 100μM, did not inhibit the metalloproteinase activity in STAUNO activated cells (red curve B) or anoikic cells (purple curve D). However, the lower panel of Figure 7 shows that the metalloproteinase inhibitor GM6001 at 50μM inhibited the metalloproteinase activity in STAUNO activated cells (red curve B) and anoikic cells (purple curve D).

The upper and lower panels of Figure 8 show serine protease activity was detected in apoptotic (blue curve A) and anoikic (green curve C) LN18 glioblastoma cells by their action upon the serine protease fluorescent substrate BOC-RVRR-AMC. Apoptosis was induced by 1μM STAUNO while the cells were in suspension. The upper panel of Figure 8 shows that the serine protease activity exhibited by LN18 cells treated with 1μM STAUNO (red curve B) and undergoing anoikis (purple curve D) were not inhibited by 50μM of the metalloproteinase inhibitor GM6001. The lower panel of Figure 8 shows that soybean trypsin inhibitor at 100μM blocks serine protease activity in STAUNO activated LN18 cells (red curve B) and anoikic cells (purple curve D). Overall the specificities of the substrates and inhibitors show that apoptotic and anoikic

![Graphs showing LN18 serine protease activity as the cells proceed through apoptosis and anoikis. Relative fluorescence generated by action of cells upon BOC-RVRR-AMC, a serine protease fluorescent substrate.](image)
glioblastoma cells demonstrate enhanced metalloproteinase and serine protease activity.

**Serine Protease Activity in Monolayer of Apoptotic LN18 Cells Inhibited by Z-VAD-FMK**

The Bar diagrams of Figure 9 show that the broad caspases inhibitor Z-Val-Ala-Asp-Fluoromethylketone (Z-VAD-FMK) blocks the apoptotic activation of serine proteases that are detectable by fluorometric substrate BOC-RVRR-AMC. The degree of serine protease inhibition is well over 50% as demonstrated by comparing the Z-VAD-FMK treated apoptotic U87-MG cells (Bar E) relative to the apoptotic U87-MG cell population not treated with Z-VAD-FMK (Bar D). Z-VAD-FMK is a cell permeable, irreversible inhibitor of caspases. Caspases are a family of cytosolic aspartate-specific cysteine proteases involved in the initiation and execution of apoptosis. It can be seen from Bar C of Figure 9 that when cells are in a monolayer and the glioblastoma cells are adhered to the extracellular matrix they are stable with regard to serine protease activation. This is in contrast to the data of Figures 7 and 8 (green curves C), which show that glioblastoma cells in suspension progress to anoikis and demonstrate considerable serine proteases activation. The Z-VAD-FMK was used at an IC50 concentration (50μM). Overall the data of Figure 9 show that apoptosis needs to progress beyond the activation of effector caspase-3 to demonstrate a full blown serine protease activity as is manifested by action upon the fluorescent substrate BOC-RVRR-AMC.

**Figure 9**: Fluorescence activity of adherent U87-MG cells acting on the substrate BOC-RVRR-AMC.

**Bar A**: 5μM of the fluorescent 7-amino methyl coumarin fluorescent standard in the absence of U87-MG cells.

**Bar B**: Fluorescence activity of control BOC-RVRR-AMC substrate in the absence of U87-MG cells.

**Bar C**: Activation of the BOC-RVRR-AMC by monolayer of non apoptotic U87-MG cells.

**Bar D**: Activation of the BOC-RVRR-AMC by apoptotic U87-MG activated by 5μM of STAURO for 8h.

**Bar E**: Activation of the BOC-RVRR-AMC by apoptotic U87-MG induced by 5μM of STAURO in the presence of 50μM caspases inhibitor Z-VAD-FMK.
A apoptotic degradation of class I histocompatibility proteins HLA-ABC

Figure 10 consists of flow cytometry histograms showing the effects upon class I histocompatibility leukocyte antigens (HLA-ABC). The histograms are displayed as counts versus the intensity of phycoerythrin conjugated secondary antibody directed against the anti-HLA-ABC primary antibodies. The histograms of Row 1 of Figure 10 illustrate the level of HLA-ABC in counts of LN18 cells (ordinate) vs. fluorescent intensity (abscissa) of three replicate control cell populations devoid of apoptotic stimulation or inhibitors. It can be seen for each sample population there is a pronounced peak in cell count at an MFI of about 6,000 units. The histograms of Row 2 of Figure 10 show the population of cells when apoptosis was induced in the LN18 cells by 50μM MK886. The decrease in the cell count at the higher fluorescence intensities indicates an apoptotic-induced degradation of the cell surface HLA-ABC determinants. Row 3 of Figure 10 shows histograms of MK886 induced-apoptosis in the presence of the enzymatic inhibitors as indicated in headings of Columns 1, 2 and 3. It can be seen in Row 3, Column 2 of Figure 10 that the metalloproteinase inhibitor GM6001 at 50μM blocks the degradation of HLA-ABC determinants that was manifested in Row 2. Row 3, Column 3 of Figure 10 shows that the serine protease inhibitor, soybean trypsin inhibitor at 100μM, similarly blocks the HLA-ABC degradation. However Row 3, Column 1 of Figure 10 shows that the inhibitor PSI at 50μM, which blocks chymotrypsin-like activity and other proteasome enzymatic activity, does not reverse the apoptotic degradation of the HLA-ABC determinants. Overall the data of Figure 10 indicate that serine proteases and metalloproteinases that are activated as the LN18 cells proceed through apoptosis are capable of degrading important immune response determinants.

Concluding Remarks

The immunosuppressive features of glioblastoma multiforme (GBM) are quite complex. What is proposed here is that cancer cells undergoing apoptosis further
complicate GBM related immune suppression. The major mechanism of radiation therapy and chemotherapeutics of cancer cells is the induction of a programmed death. An early event in the modality of programmed cell death is the externalization of phosphatidylserine (PS). PS exposure is a signal for phagocytic cells to remove dead and dying cells by the process of efferocytosis, which minimizes necrotic cell induced inflammation. Although apoptosis is the major mechanism in the elimination of malignant cancer cells, and considered beneficial, it can have negative effects. For example, externalization of PS can be immunosuppressive [90] and the apoptotic process can enhance the invasiveness of non-apoptotic malignant cells [78].

A main focus of this chapter is to demonstrate that apoptotic glioblastoma cells have very different characteristics than non-apoptotic glioblastoma cells. There is the general understanding that glioblastoma patients are immune suppressed. In this present chapter attention was called to the heretofore-unrealized possibility that the enzyme activating characteristics of the apoptotic cells within the glioma tumor could significantly contribute to the patient's immune suppression. It was demonstrated that glioblastoma cells possess ADAMs, furin and matriptase proteases that are significantly activated in apoptotic as compared to non-apoptotic glioblastoma cells. The apoptotic glioblastoma cells were also shown to have the capacity to degrade important immune molecules such as the cell surface determinants HLA-ABC and soluble proteins such as C3a that could be reversed by metalloproteinase and serine protease inhibitors.

Immunotherapy, as an approach to the treatment of cancers, dates back to the late 1960s and early 1970s when attempts were made to capitalize on advances made in the understanding of B and T cell types of the immune system [91,92]. Subsequently, the discovery of cell surface histocompatibility determinants [93] and the identification cytokines and their effects [94] increased interest in immunotherapeutic approaches to cancer treatment [95]. Until very recently, the promise of immunotherapy in the treatment of cancer was not realized. However, there currently are explorations of different types of immune therapy in the treatment of glioblastoma multiforme including: cytokine-stimulated natural killer cells as adjuvant therapy [96], autologous natural killer cell therapy [97], chimeric antigen receptor (CAR) engineered natural killer cells and CAR engineered T cells [98-100].

The CAR-T cell technologies is a revolution in immune therapy that is rapidly developing due to new techniques of editing the genome of immune cells by conveniently inserting specific DNA sequences as part of the gene that codes for cytotoxic T cell and natural killer cell receptors. This technique is based on the use of clustered regularly interspaced short palindromic repeats (CRISPR) that allows the convenient insertion of DNA sequences within specific alleles of a chromosome. In other words,
in a CAR-T cell DNA that codes for a surface antigen of the target cell is inserted at the specific locus that codes for the T cell receptor. This produces a chimeric receptor part of which binds an antigen determinant on the cancer cell and part of which positions the T cell to be activated to kill the targeted tumor cell. Recent advances in tissue culture techniques accompany CRISPR technology. This enables taking a small population of cytotoxic T cells from the patient, genetically modifying them by CRISPR, and subsequently culturing them in vitro to very high numbers of CAR-T cells. The high population of CAR-T cells is then infused into the patient as immunotherapy.

Immunotherapeutic approaches in the treatment of cancers show great promise. However the proteolytic removal of surface determinants of the target cell, and other cells essential to the efficacy of the immune process, can be immunosuppressive and the removing of surface antigens can also interfere with the effectiveness of CAR-T cell therapy. Thus, the immunosuppressive effects of high numbers of apoptotic cells needs to be considered if a total understanding of immunotherapy as a treatment of malignancies is to be achieved.

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