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INDUCTION OF T CELL APOPTOSIS BY GLIOBLASTOMA MULTIFORME

Keith Zachary Sabin
Northern Michigan University

INDUCTION OF T CELL APOPTOSIS BY GLIOBLASTOMA MULTIFORME

By

Keith Zachary Sabin

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_____
Committee Chair: Dr. Robert J. Winn, Ph.D.   Date

_____
First Reader: Dr. Robert J. Belton, Ph.D.   Date

_____
Second Reader: Dr. Erich N. Ottem, Ph.D.   Date

_____
Third Reader: Dr. Richard A. Rovin, M.D.   Date

_____
Department Head: Dr. John E. Rebers, Ph.D.   Date

_____
Dr. Brian D. Cherry, Ph.D.   Date
Assistant Provost of Graduate Education and Research

ABSTRACT

INDUCTION OF T CELL APOPTOSIS BY GLIOBLASTOMA MULTIFORME

By

Keith Zachary Sabin

Glioblastoma multiforme is the most common and malignant brain tumor in adults. Despite an intensive treatment regimen the average survival after diagnosis remains only 15 months. Novel therapies are desperately needed. While recent successes with immune based therapies, in targeting brain tumors, are promising widespread clinical efficacy has yet to be demonstrated. Glioblastoma patients are systemically immune suppressed and to increase the effectiveness of immune based therapies these immunosuppressive pathways must be neutralized. A possible role for glioblastoma-derived exosomes in immune suppression has never been investigated. Exosomes are small membrane-bound vesicles of endocytic origins that contain mRNA and proteins and are secreted by a variety of cell types, including brain tumors. We have shown that glioblastoma-derived exosomes are capable of decreasing T cell viability. In order to delineate the signaling pathway responsible for reduced T cell viability a Fas neutralizing antibody was utilized which yielded enigmatic results. However, glioblastoma-derived exosomes activated a caspase cascade indicative of Fas signaling and the activation of the extrinsic apoptotic pathway. This is a novel form of immune suppression in glioblastoma and could lead to the development of novel therapies.

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Glioblastoma multiforme (GBM), also known as a grade IV astrocytoma, is the most common and malignant primary brain tumor in adults. Hallmarks of GBM include its heightened proliferative potential and extensive neovascularization. The ability of GBM to proliferate so rapidly is due, in part, to the inactivation of the major tumor suppressor genes retinoblastoma protein (pRb)[1] and phosphatase and tensin homologue (PTEN)[2]. The inactivation of these tumor suppressors allows for an uninhibited progression through cell cycle checkpoints. GBM is highly vascularized due to increased expression of the pro-angiogenic molecules stromal derived factor-1 (SDF-1), fibroblast growth factor (FGF), interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF)[3]. Neovascularization of the tumor is such a defining characteristic of GBM that it prompted investigations into whether or not blood vessel density could be used as a prognostic indicator. As it turns out, the extent of vascularization proved to be inversely correlated with mean survival in patients with astroglial tumors[4,5].

The current gold standard of treatment for GBM patients includes maximal surgical resection coupled with radiation and chemotherapy. The current chemotherapy most widely administered to patients with newly diagnosed GBM is the alkylating agent temozolomide (TMZ)[6]. TMZ acts by adding a methyl group to the O^6 position on the nucleotide, guanine. This methyl adduct inhibits the ability of the cancer cell to correctly replicate its DNA. Base excision repair pathways are activated to replace the methylguanine with a guanine. However, TMZ methylates the O^6 position of the new nucleotide leading to activation of base excision repair pathways yet again. This leads to

<center>1</center>

single stranded DNA breaks and futile mismatch repair cycles inevitably leading to cell death (Figure 1)[7]. A major mechanism of TMZ resistance in GBM is mediated by the expression of the enzyme O^6-methylguanine-methyltransferase (MGMT). MGMT specifically recognizes O^6 methyl adducts on guanine and removes them[7]. MGMT effectively neutralizes the cytotoxic abilities of TMZ. It was shown that patients with a methylated MGMT promoter, which effectively silences gene expression, respond better to TMZ treatment than patients with an unmethylated MGMT promoter[8]. However, despite this aggressive multi-modality treatment regimen the average survival rate after diagnosis is only 15 months with a 5 year survival rate of less than 4%[6,9]. Novel treatments are desperately needed and recently increased efforts have gone into the development of immune based treatment of malignant gliomas.
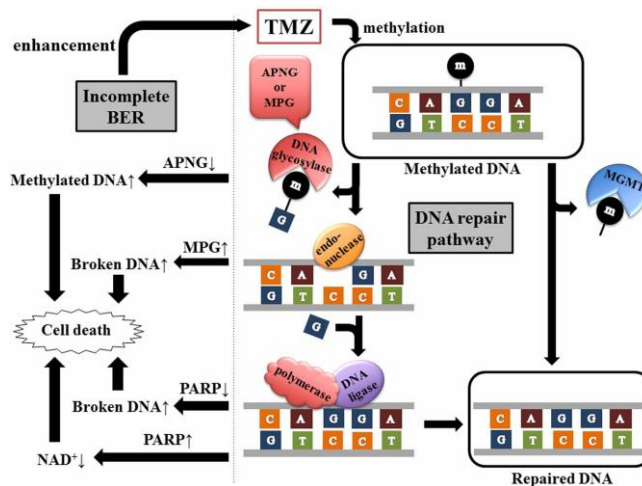
Figure 1: Mechanism of MGMT mediated Chemoresistance. TMZ generates a spectrum of DNA lesions including O6-methylguanine, N3-methyladenine and N7-methylguanine. MGMT directly eliminates the methyl group from O6-methylguanine, whereas base excision repair pathway includes multistep reaction by DNA glycosylase (APNGorMPG), endonuclease, polyeraswe and DNA ligase. DNA glycosylase recognizes and removes the damaged bases. The abasic site is then hydrolyzed by endonuclease, resulting in the incision of the damaged DNA strand. Polymerase inserts a single nucleotide and DNA ligase completes the repair process. Poly (ADP) ribose polymerase, one of the polymerases of the base excision repair pathway, catalyzes the transfer of ADP-ribose units from NAD+ to target proteins including PARP itself. Therefore, inhibition or hyperactivation of PARP leads to accumulation of broken DNA or NAD+ depletion respectively, consequently inducing cell death.

The immune system in patients that develop tumors has already failed. The immune system is capable of recognizing a cancerous cell and destroying it. However, in cancer patients, the immune system is sufficiently suppressed to the point that it is unable to mount a robust antitumor response without clinical intervention. Components of the immune system are being used in clinical settings to help manage different disease states, and these fall into two broad categories: passsive immunotherapy and active immunotherapy.

Passive immunotherapies include the use of antibodies, cytokines and antigen unrestricted lymphocytes[10]. One monoclonal antibody whose clinical relevance is being investigated in GBM is bevacizumab[11–13]. Bevacizumab is a monoclonal antibody recognizes the pro-angiogenic molecule VEGF, which, as discussed earlier, plays an important role in glioma progression. The antibody binds to VEGF in such a way that it inhibits its ability to bind its cognate receptor, thereby antagonizing the formation of new blood vessels[13]. Clinicians have also investigated using injections of potent immune stimulatory cytokines, such as interleukin(IL)-2. This approach, in theory, is thought to help lymphocytes overcome tumor-derived, immunosuppressive factors and stimulate them to proliferate and subsequently differentiate into effector and memory cells. IL-2 therapy has been successful in melanoma and renal cell carcinoma[14] but its clinical efficacy has not been demonstrated in the case of GMB[15]. Another method involving passive immunotherapy that has been utilized in the treatment of GBM includes the *ex vivo* activation of tumor infiltrating lymphocytes (TILs) and subsequent infusion of the activated lymphocytes back into the patient[16–18]. It is proposed that lymphocytes found within the tumor are more likely to be reactive to tumor antigens. The lymphocytes are

activated *ex vivo* then reintroduced into the patient and should be able to mount a robust antitumor immune response.

Active immunotherapies involve the *in vivo* stimulation of a patient's immune system to mount an antigen-specific antitumor attack. The most common forms of active immunotherapies utilize peptide-based vaccines or employ the antigen presenting capabilities of dendritic cells[10]. Both treatment modalities have been and continue to be investigated in the treatment of GBM. One of the biggest obstacles of advancing active immunotherapies is finding an antigen that is tumor specific and not expressed by healthy cells. A very attractive tumor specific antigen found in GBM is epidermal growth factor receptor variant III (EGFRvIII). EGFRvIII is a constitutively active, truncated receptor that lacks the extracellular ligand binding domain. It is a common antigen in GBM that is not expressed elsewhere in the body. The mutation responsible for its constitutive activation causes a novel sequence of amino acids to be exposed on the extracellular surface of the cell. This epitope has been mapped and developed into a peptide based vaccine[19–22]. The injection of this specific peptide into patient with EGFRvIII positive tumors is sufficient to evoke a robust and antigen specific antitumor immune response in a cohort of patients[21,22].

Another commonly used form of active immunotherapy involves the isolation of patient monocytes which are then differentiated into dendritic cells *ex vivo*. These patient-derived dendritic cells can be pulsed with autologous tumor lysate and reintroduced into the patient. Once in the patient, the dendritic cells appropriately process the tumor antigens and display them to the immune system and initiate an antitumor immune response[23–26]. While success with immune-based therapies has been reported in

GBM studies, they are rare, with only modest improvements to current survival rates. These results differ from reports from other types of cancer including melanoma and renal cell carcinoma which respond well to certain types of immunotherapies[14,15]. This leads to the question: What is it about the nature of GBM that makes it refractory to immune based therapies?

One potential answer to this question is simply the location of the tumor. Gliomas arise in the brain and rarely ever metastasize from the central nervous system (CNS). The brain has long been considered an immune privileged organ that is ill suited to initiate or maintain an immune response. Therefore, addition of immune cells to the tumor cavity or to the periphery should have little influence on tumor cells residing within this immune privileged niche. However, recent evidence suggests that the brain is far from the immune-isolated organ that it was once thought to be, and as such, provides an environment conducive to the formation and persistence of an antitumor immune response. An alternative answer, that is likely underappreciated, is the degree of immune suppression afforded by the tumor both locally, in the tumor microenvironment, and systemically. Both the immune status of the CNS and immunosuppressive mechanisms employed by GBM will be discussed presently.

For decades, the brain was considered an immune privileged organ for several reasons: (1) there was no apparent draining lymph node from the CNS, (2) it was thought the blood brain barrier (BBB) provided an absolute barrier to circulating peripheral leukocytes and (3) the relatively low numbers of antigen presenting cells (APCs) within the CNS was not conducive to the stimulation of an immune response. These notions have recently been disavowed.

Several studies have shown that cerebrospinal fluid (CSF) can drain through the Virchow-Robbin spaces to the deep cervical lymphatics[27–29]. One of the first indicators that antigens from the CNS could stimulate an immune response was provided by microinjection of human serum albumin (HSA) into the CSF of rats[29]. The injection of HSA into the CSF of rats was sufficient to elicit anti-HSA antibody production with the increased antibody titer persisting over 10 weeks[29]. Obstruction of the cervical lymph nodes was able to inhibit the formation of HSA reactive antibodies[29]. Further support that CNS antigens are capable of entering the lymphatics was provided by studies utilizing radiolabeled albumin. The labeled proteins were microinjected into the CSF or brain of rabbits, cats and sheep, and they were able to trace the radiolabeled protein as they migrated from the CNS into lymph[28]. These studies provide compelling evidence that soluble antigens from the CNS are capable of entering into the lymphatics and are able to stimulate humoral and cellular immune responses.

Additional studies sought to characterize the ability of T lymphocytes to cross the BBB and enter into the CNS. Naïve T cells were unable to enter into the CNS but activated T cells are capable of extravasation across the BBB and gaining entrance to the CNS[30,31]. Activated T cells are capable of crossing the BBB via interactions between cell adhesion molecules found specifically on activated T cells and on the brain vasculature[31]. Specifically, it appeared that alpha 4 integrins were essential to T cell trafficking into the CNS. These studies also demonstrated that activated T cells are capable of entering the CNS, independent of antigen specificity, and can then reenter circulation to patrol other organs. These data indicate the ability of T cells to enter the CNS is dependent on their state of activation, as opposed to antigen specificity[30].

Another reason the CNS was thought to be an inhospitable environment for the formation of an immune response was the relative lack of professional APCs. APCs are a specialized type of immune cells that are responsible for stimulating antigen specific immune responses. It is now clear that CNS accessory cells called microglia, which arise from bone marrow-derived cells, are fully competent to act as resident macrophages in the CNS[32]. Functionally and phenotypically, microglia are capable of processing and presenting antigens in a manner similar to the dendritic cells of the periphery[32–34]. They express class II major histocompatibility complex and the appropriate co-stimulatory molecules necessary for antigen presentation and activation of T lymphocytes[35]. In experiments using primary microglia cultures, it was shown that these cells were sufficient for activation of T cells both *in vitro* and *in vivo*[34,36].

Taken together, these lines of evidence dispel the notion of the brain as an "immune privileged" organ. Antigens from the CNS are capable of entering into the lymphatics system and eliciting a measurable and sustained humoral and cellular response[27–29]. Studies demonstrate that activated T cells are capable of crossing the BBB, and participate in an immune response[30,31]. CNS accessory cells in the form of microglia function as APCs that express the necessary MHC molecules and co-stimulatory factors necessary for the formation of a successful immune response[32–36].

However, while the CNS has all the hallmarks of an immune competent organ it is important to note that, while the BBB does not form an absolute barrier to leukocytes it does seem to prevent the excursion of naïve T lymphocytes into the brain parenchyma[31]. Taking this into account, it is probably more accurate to refer to the CNS as an "immune-restricted" organ. Passive incursions by circulating naïve lymphocytes into the brain

parenchyma seem to be restricted, presumably by the presence of the BBB, but activated lymphocytes are fully capable of circumventing the BBB. What, then, is the nature of GBM-derived immune suppression?

It's been more than 40 years since the first observations indicating that cellular immunity in glioma patients was suppressed[37,38]. This state of immune suppression is absent in patients prior to tumor formation. Studies investigating the functionality of cellular immune responses in patients pre- and post-tumor resection indicated that these deficiencies are reversed after tumor resection but return upon recurrence[39]. The underlying mechanisms responsible for decreased cellular functionality have been extensively investigated[40–42].

Lymphocytes isolated from peripheral circulation of GBM patients exhibit extensive abnormalities in their ability to undergo clonal expansion *in vitro* in response to mitogen stimulation[42]. It was shown that the inability of T cells isolated from GBM patients to undergo proliferation was due to defects in the IL-2/IL-2 receptor (IL-2R) pathway[43,44]. The IL-2 receptor is comprised of three polypeptides, designated the α- β- and γ- chain[45]. Naïve T cells express an intermediate affinity IL-2R comprised of a β-chain and a γ-chain. The expression of the high affinity IL-2R is necessary for clonal expansion and subsequent T cell activation[46–48]. Following antigenic stimulation, T cells begin to express the α-chain, which is required for the formation of the high affinity IL-2R[45,46,49]. T cells obtained from GBM patients did not express the high affinity IL-2R[50] and this deficiency was later demonstrated to be in response to a tumor-derived immunosuppressive factor[51]. Further supportive evidence for the existence of a soluble tumor-derived immunosuppressive factor capable of mediating these effects was

provided by experiments that cultured primary T cells from healthy individuals in glioma-conditioned medium. In response to activating stimuli these T cells exhibited similar deficiencies in IL-2 secretion and lacked expression of the high affinity IL-2R, CD25, indicating a role for a tumor specific soluble immunosuppressive factor in T cell inhibition[52–54].

Further studies aimed at characterizing the soluble immunosuppressive factor responsible for these T cell deficiencies identified transforming growth factor (TGF)-β2[55,56]. Eventually expression of all three isoforms of TGF-β was demonstrated for GBM[57,58]. The potent immune suppressive role of TGF-β was demonstrated for melanoma using mice genetically engineered to have T cells express a dominant negative, or inactive, form of the TGF-β receptor[59]. Mice whose T cells expressed the nonfunctional dnTGF-β receptor were able to reject the tumor, while wild type mice, who expressed a functional TGF-β receptor, were unable to reject the tumor and succumbed to tumor burden[59]. The physiologic consequences of TGF-β expression by GBM takes on additional significance in light of recent findings that TGF-β is capable of inducing peripheral CD4+ helper cells to become phenotypically and functionally regulatory T cells[60,61].

Regulatory T cells (Tregs) are a subset of T cells phenotypically defined by the expression of CD4, CD25 and the foxhead transcription factor, FoxP3[62]. Tregs are capable of inhibiting effector T cell function in a variety of ways including: (1) TGF-β induced immune suppression, (2) release of the immunosuppressive cytokine IL-10 and (3) ability to release the immunosuppressive factor adenosine[62–64]. Recently, an increased presence of Tregs in the tumor was indicated as a poor prognostic indicator for cervical

cancer and GBM[65,66]. Another immunosuppressive mechanism employed by GBM is the expression of the pro-apoptotic molecule Fas ligand (FasL).

FasL is capable of binding to its receptor, Fas, and activating the intrinsic apoptotic pathway which results in completion of programmed cell death. This pathway will be discussed in more detail later. The Fas/FasL system is thought to be an important pathway involved with the homeostatic regulation of immune responses. Activated T cells constitutively express Fas which is thought to be a key mediator of activation induced cell death (AICD). AICD functions as the brakes to an immune response and helps to ensure that an inappropriate immune response does not occur. The physiologic importance of this pathway becomes evident in individuals who have autoimmune lymphoproliferative disease (ALPS). While there are several different classifications of ALPS, they all result from different mutations to the *FAS* gene. These mutations are all loss of function and results in lymphocyte resistance to FasL-induced apoptosis[67]. This resistance to FasL-mediated apoptosis in those T cells allows for massive proliferation of lymphocytes and the development of inappropriate immune response. While FasL-induced apoptosis is a physiologic function, this mechanism is exploited by a number of tumor types including GBM as a potent immune suppressive mechanism[68]. The overexpression of FasL is a necessary event early in the carcinogenesis of colon cancer[69]. The functional significance of FasL expression has been demonstrated for GBM *in vitro* and *in vivo.*

FasL expression was confirmed in freshly resected tumors and established cell lines using Western blotting[68]. When GBM cells were co-cultured with T cells, it was sufficient to stimulate apoptosis in the T cells. The necessity of FasL to induce apoptosis

was demonstrated by the ability of a FasL neutralizing antibody to rescue T cells from apoptosis in the co-culture assay[68]. When GBM cells, engineered to no longer express FasL, were injected into immune competent rats, the resulting tumor grew significantly slower and had an increased presence of tumor infiltrating lymphocytes, as compared to tumors generated from wild type GBM cells[68].

It is apparent that patients with GBM exhibit severe immune suppression, both in the tumor microenvironment and in the periphery. This immune suppression is mediated by a number of membrane bound factors, such as the pro-apoptotic molecule FasL, and soluble secreted factors, like TGF-β[57,68]. It is clear that in order for future immune-based therapies to mount a robust and reproducible anti-tumor effect, the immune suppressive pathways employed by the tumor need to be better understood. While the above-mentioned pathways are under intensive experimental investigation, the role tumor-derived exosomes play in immune suppression remains largely uncharacterized.

<center>Exosomes: Biogenesis and Function</center>

In order to communicate, cells secrete factors into the extracellular environment. These factors are a diverse group of signaling components, ranging from macromolecular complexes, to soluble proteins, to modified fatty acid chains. These secreted factors are capable of influencing the behavior of neighboring cells in response to a specific or a series of stimuli. One mode of cell-to-cell communication that is receiving increased attention is one which is mediated by exosomes. Exosomes are small (50-100 nm) membrane-bound vesicles of endocytic origin that are released by a diverse array of cell types, including cancer cells. Exosomes contain membrane and soluble proteins, as well

as m RNA and miRNA[70,71]. An overview of the exosomal pathway is depicted in Figure 2[72].
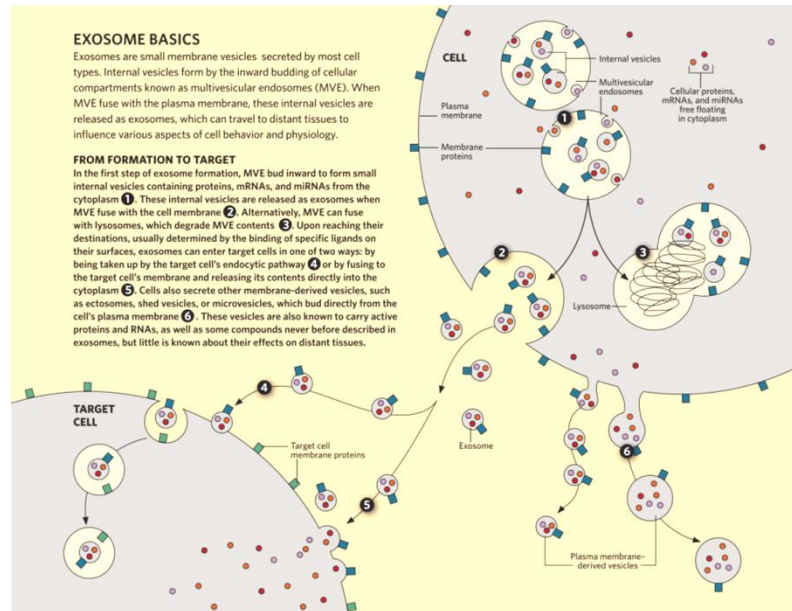
Figure 2: An overview of the Exosomal Pathway. Exosomes correspond to the intraluminal vesicles (ILV) found in multivesicular bodies (MVB). Mature MVB can either enter the lysosomal pathway resulting in the degradation of its contents or it can fuse with the plasma membrane thus releasing the ILV as exosomes into the extracellular environment. Exosomes contain membrane proteins, soluble proteins, mRNA and miRNA. Once in the extracellular environment exosomes can either directly fuse with the membranes of recipient cells or can be internalized through endocytosis. The physiology of the recipient cell will be appropriately influenced by the molecular composition of the exosome.

Exosomes were first described as "exfoliated vesicles" that possessed ectoenzyme activity and were capable of acting as a 5' exonucleotidase[73]. Studies performed several years later were able to show that sheep reticulocytes shed transferrin receptors as they differentiated in similar structures[74,75]. Since these reports, much has been learned about the origin of these structures. Exosomes correspond to the intraluminal vesicles (ILV) present in multivesicular bodies (MVBs)[76–78]. ILV arise from the invagination of the limiting membrane of early endosomes and the accumulation of ILV leads to the formation of MVB. At this point, MVBs enter one of two pathways. They either fuse

with lysosomes and its contents are degraded, or they fuse with the plasma membrane and release the ILV as exosomes[79].

Formation of ILV is not well understood, but increasing evidence suggests that clustering of proteins in the limiting membrane of the early endosome promotes membrane invagination and ILV creation. Experimental evidence utilizing crosslinking antibodies or aceylated reporter proteins support this notion. Inducing the aggregation of the transferrin receptor in maturing rat reticulocytes or of major histocompatibility complex class II (MHC class II) complexes in activated B cells via crosslinking antibodies increased their localization to the endosomal compartment and subsequent secretion in exosomes[80,81]. Consistent with these results, the presence of multiple oligomerization domains in an acylated reporter protein increased the presence of the reporter protein in exosomes[82]. Taken together these data demonstrate a role for cargo clustering in ILV formation.

Another mechanism that seems to play a role in the formation of ILV involves the endosomal sorting complex required for transport (ESCRT) proteins. There are four predominant members of the ESCRT complex: ESCRT-0, -I, -II, -III[83]. The function of ESCRT-0, -I and -II involve interacting with and retaining ubiquitinated proteins in the endosomal compartment, while ESRCT-III plays a role in membrane budding[84,85]. The interactions of ESCRT-0, -I and -II with ubiquitinated proteins could indirectly stimulate ILV formation by promoting the clustering of ubiquitinated proteins in the limiting endosomal membrane. Previous studies indicated that protein clustering promoted their release in exosomes[80–82]. The action of ESCRT-III could directly promote ILV formation through the stimulation of membrane budding of the early endosomal membrane. While

13

evidence suggests a role of ESCRT-dependent ILV formation, it appears that ILV can form in an ESCRT-independent mechanism.

Recent experimental evidence suggests that the accumulation of the sphingomyelin, ceramide, plays a role in ILV formation and the promotion of exosome secretion. Studies focusing on the biochemical composition of exosome-associated lipids found that cholesterol, ceramide and its derivative hexosylceramide, were enriched in the membranes of exosomes when compared to the parent cell[86]. The enzyme neural sphingomyelinase (nSMase) is responsible for the production of ceramide. When nSMase function was inhibited either through pharmacological means or small interfering RNA (siRNA) applications, ceramide was drastically reduced in the parent cells and the amount of exosomes being released was significantly decreased[86,87]. Furthermore, transfecting cells with a vector encoding nSMase, thereby increasing the expression levels of this enzyme, caused an increase in exosome secretion[87]. This increase in exosomes secretion was independent of ESCRT expression[87]. Further investigation will be required to determine the pathways necessary for ILV formation and the regulation of MVB fate.

Another category of membranous vesicles released by cells is referred to as microvesicles (MV). MV (100-1,000nm) are larger than exosomes (50-100nm) and are the result of an independent biosynthetic pathway. They arise from the direct budding of the plasma membrane[88] via a mechanism dependent on increased calcium permeability[89–91] and the activation of calpain[92]. More extensive characterization of the contents of MV indicate that they are enriched with lipid raft-associated molecules and cholesterol[90,91]. While the biosynthetic pathways and molecular composition of Mvs may differ from

exosomes, the ability of these different populations of membranous vesicles to diffuse away from the cell of origin and influence the physiology of recipient cells is becoming increasingly appreciated[93,94]. Studies utilizing exosomes isolated from cancer cells have greatly contributed to the understanding the role exosomes could play in cancer progression.

Tumor-derived exosomes potentially contribute to tumor progression by modulating a variety of pathways including immune suppression, cell proliferation, angiogenesis and metastasis[95–98]. As previously mentioned, a common oncogenic receptor expressed by GBM cells is the truncated, constitutively active EGFRvIII. Exosomes isolated from established GBM cells lines that express EGFRvIII contain functional receptor and are able to transfer the receptor to cells that do not normally express EGFRvIII[95,96]. Exosomal-mediated transfer of the receptor led to a significant increase in proliferation, of the recipient cells. This response was specific to the transfer of EGFRvIII as exosomes isolated from GBM cells lines that did not express the oncogenic receptor were unable to significantly increase proliferation[95,96]. In addition to increased proliferation the exosomal mediated transfer of EGFRvIII was shown to increase the secretion of the pro-angiogenic protein VEGF, induced activation of the Akt and MAPK/Erk pathway, upregulated the expression of the anti-apoptotic protein Bcl-x_L and down regulated the expression of p21^{cip1}, an inhibitor of cell cycle progression[27].

A second study using GBM-derived exosomes corroborated the ability of EGFRvIII containing exosomes to transfer a functional receptor and increase the proliferation of recipient cells[96]. In addition, the composition and functional implications of mRNA extracted from GBM-derived exosomes was also investigated. Using

microarray technology it was determined that approximately 4,700 transcripts were specifically enriched in the exosome fraction and absent from the parent cells[96]. The functions of these mRNA were investigated using bioinformatic analysis, and the most common function of the enriched mRNA was involved in cell proliferation pathways, immune response, angiogenesis and migration[96]. Interestingly, the mRNA composition in exosomes isolated from two different tumor samples poorly correlated with the mRNA composition of the respective parent cells, while the mRNA composition of the exosome samples or the parent cells was strongly correlated[96]. These data suggest a conserved mechanism for the specific enrichment of certain mRNAs into GBM-derived exosomes. This is particularly interesting when considering that the specifically enriched mRNA contribute to pro-tumor pathways such as proliferation and angiogenesis.

Further evidence suggesting tumor-derive exosomes contribute to angiogenesis comes from a study evaluating the composition of mRNA from colorectal cancer cell-derived exosomes[98]. Using microarray technology 241 mRNAs were found to be enriched above levels determined for the parent cells. Of those 241 transcripts, 27 were found to be involved with cell cycle progression, specifically for M-phase progression[98]. When colorectal cancer-derived exosomes were incubated with endothelial cells, this was sufficient to increase the proportion of cells undergoing mitosis[98] thus providing evidence that exosomes contribute to angiogenesis by promoting the division of endothelial cells.

A role for tumor-derived exosomes has also been implicated in immune suppression. Exosomes isolated from a diverse set of tumor types including melanoma, colorectal cancer and prostate cancer are capable of inducing T cell apoptosis via a Fas ligand (FasL) dependent mechanism[99–101]. FasL stimulates programmed cell death, or

apoptosis, by binding to its receptor, Fas, and initiating a caspase cascade in the Fas-expressing cell. FasL is often over expressed in tumor cells, including GBM[102,103]. Exosomes isolated from cultured cancer cell lines and incubated with either established T cell lines[99,100], activated, or resting primary peripheral blood mononuclear cells (PBMCs)[100] or primary CD8+ cytotoxic T cells[100,101] were capable of inducing apoptosis in all cells tested. However, the ability of tumor-derived exosomes to induce apoptosis was restricted to cells that expressed Fas. Furthermore, addition of a Fas neutralizing[99,100] or a FasL neutralizing[101] antibody was sufficient to inhibit the ability of tumor-derived exosomes to induce apoptosis. It has been shown that GBM cells express FasL[102] however the presence of FasL in GBM-derived exosomes has not been reported.

Apoptosis: Fas ligand/Fas/DISC/Caspase-8

Programmed cell death, or apoptosis, is a tightly regulated physiologic function. In 1842, Carl Vogt was the first to propose the idea that cells are capable of undergoing natural cell death. The first documentation of the morphological changes associated with this natural cell death program was provided by Walther Flemming in 1885 and in 1972 the term apoptosis was first proposed[104,105]. As our understanding of apoptosis grew, cancer researchers desperately searched for ways to exploit this pathway for the successful treatment of cancer. Several researchers sought to develop monoclonal antibodies that were capable of activating the apoptotic program in both malignant and normal cells[106,107]. It was later determined that two of the most potent monoclonal antibodies developed, anti-APO-1 and anti-Fas, were capable of recognizing similar epitopes and activating the same cell surface antigen. Subsequent cloning experiments determined that the antigen recognized by the antibodies belonged to the Tumor Necrosis

Factor Receptor superfamily (TNFRSF); specifically the pro-apoptotic cell surface receptor, Fas[108]. Other members of the TNFRSF include TNF receptor, CD40 and TNF-related apoptosis inducing-ligand receptor (TRAIL-R)[109].

TNF receptors are type I transmembrane receptors, meaning the N-terminus is oriented on the extracellular surface, and there is one transmembrane domain and the C-terminus is oriented on the intracellular surface[108,110]. A defining hallmark of TNFRs is the presence of several cysteine-rich domains (CRD) in their extracellular domain. Depending on the receptor, there can be up to six CRDs. The CRDs are capable of forming intrapeptide disulfide bonds which are indispensable for receptor stability and function[110]. The CRDs play a crucial role in ligand recognition, binding and receptor subunit pre-association[110,111].

While the TNFRSF encompasses many different receptors that are involved in a variety of cellular processes, there is a subgroup of TNFRSF members referred to as the death receptors (DR). Eight members of the DR subgroup have been characterized: TNFR1, Fas, DR3, TRAILR1, TRAILR2, DR6, ectodysplasin A receptor (EDAR), and nerve growth factor receptor (NGFR)[112]. These receptors were initially characterized by their ability to induce programmed cell death in response to ligand binding and receptor activation. While the biochemical structure of the receptors varies, one unifying feature of the DRs is the presence of a highly conserved sequence consisting of 80 amino acids located in the cytoplasmic tail[113]. This conserved sequence is referred to as the death domain (DD) and mediates homotypic interactions with other DD containing proteins. A functional DD is essential for the recruitment of the appropriate protein complexes to initiate apoptosis. While there are several members of the DR subgroup, Fas is

considered the prototypic DR and therefore it's structure and signaling cascade have been and continue to be the focus of intensive research efforts[109,114–116].

Since Fas was cloned in 1991, extensive studies have aimed at investigating the *FAS* gene organization and protein structure. The *FAS* gene is located on chromosome 10q24.1, includes 9 exons and spans 26kb of DNA[117]. The mature Fas transcript codes for a type I transmembrane receptor of 319 amino acids. The extracellular domain consists of amino acids 1-157 and contains three CRDs. The intracellular domain consists of 145 amino acids and contains the DD common to all DR (Figure 3)[115].
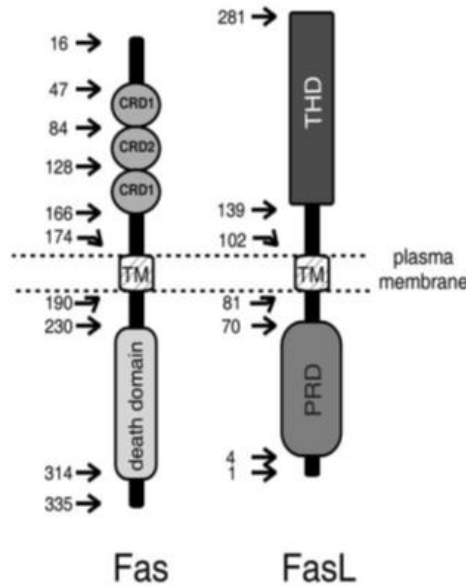
Figure 3: A Schematic Diagram of Fas and FasL Structure. The structure of Fas and FasL and the location of relevant domains. The numbers indicate amino acid positions. TM = transmembrane, PRD = proline rich domain, CRD = cysteine rich domain, THD = TNF homology domain.

The ligand responsible for activating Fas belongs to the Tumor Necrosis Factor family of ligands and is called Fas ligand (FasL). The *FASL* gene is located on chromosome 1q23, spans approximately 8kb of DNA and contains four exons[118]. Similar

to a majority of TNF ligands the *FASL* gene encodes a type II transmembrane glycoprotein[119]. The extracellular C-terminus of FasL contains three potential N-glycosylation sites[119,120] and is assembled in a "jelly roll" structure comprised of two antiparallel β-sheets[120,121]. The "jelly roll" structure is a common motif found in TNF family ligands and is therefore known as TNF homology domain (THD); (Figure 3)[115,121]. Interestingly, studies looking at the amino acid sequences of different TNF ligands have shown that sequence homology at the THD is no greater than 35%. It turns out, the conserved tertiary structure of the THD mediates ligand homotrimerization and receptor binding[122].

Direct protein-protein interactions between FasL and Fas were mapped to CRD2 and CRD3 found in the extracellular region of Fas. No direct ligand/receptor interactions were found for CRD1[123,124]. However, while there are no direct interactions between FasL and CRD1 the structural integrity of CRD1 is important for receptor signaling. When CRD1 is mutated or absent, it is sufficient to severely inhibit the ability of Fas to induce apoptosis in response to ligand binding[124]. It would appear that while CRD1 does not participate in ligand binding, it is indispensable for receptor activation and subsequent signaling.

A possible explanation for these disparate results came with the discovery that in order to be fully functional several members of the TNFRSF (TNF receptor, CD40 and TRAIL-R1) need to pre-assemble into homotrimeric complexes. The formation of receptor complexes is independent of ligand binding and if the homotypic interactions mediating subunit trimerization were interrupted the receptors were nonfunctional[125]. The corresponding domain was named the pre-ligand association domain (PLAD). While this

study looked at several members of the TNFRSF it did not directly investigate Fas signaling. However, given the previous experimental data correlating CRD1 integrity and the ability of Fas to activate apoptosos[123,124] it seems plausible that Fas could behave in a similar manner.

In order to investigate if Fas signaling was regulated by a similar mechanism, experiments utilizing yeast two-hybrid and Forrester's resonance energy transfer experiments were performed[67,126]. As it turns out, when CRD1 was mutated or deleted Fas subunits were unable to oligimerize. The inability of Fas subunits to interact with each other directly inhibited its ability to induce apoptosis[67,126]. Recent studies have precisely mapped the region of Fas necessary for subunit association and found the minimal amino acid sequence required for subunit trimerization resides between amino acids 43-66[127], which overlaps with CRD1 (amino acids 47-84)[115]. Taken together, these lines of evidence indicate that upon mutation of PLAD, Fas subunits are unable to form homotrimeric complexes. The inability of Fas to pre-assembly directly abrogates its ability to induce apoptosis.

While early studies using agonistic antibodies were capable of showing that Fas signaling leads to apoptosis[106,107], the biochemical pathway that Fas activated remained elusive. Initial studies targeting Fas used co-immunoprecipitation to show that in response to receptor activation a high molecular weight, multi-protein complex was associated with Fas[128]. When Fas constructs that lacked or had a nonfunctional DD were expressed in primary and established cell lines, the formation of this multi-protein complex was not observed. Expression of these mutant Fas constructs rendered the cell lines insensitive to Fas-induced apoptosis[128]. These findings were consistent with

previous experiments that demonstrated that the DD was necessary for Fas-induced apoptosis[129]. Taken together, these data indicated that a functional DD is required for the formation of a multi-protein complex which is directly responsible for the transduction of the apoptotic signal. As a result, this multi-protein complex was named the death inducing signaling complex or DISC[128]. While there are many proteins capable of forming the DISC the four most abundant proteins are Fas Associated Death Domain (FADD), procaspase-8 (also known as FLICE), procaspase-10 and FLICE-like Inhibitory Protein (c-FLIP) (Figure 4)[109,130–134]. The protein c-FLIP has anti-apoptotic functions and will not be discussed in this review.
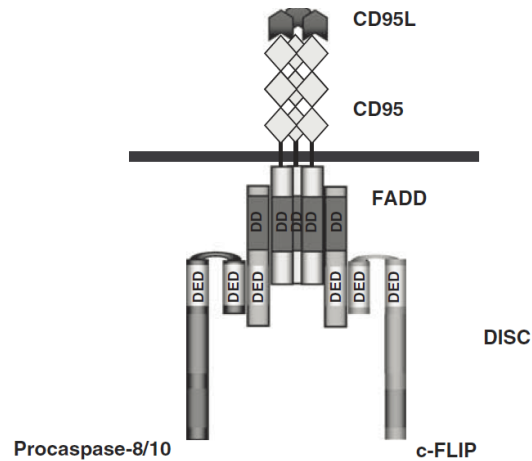
Figure 4: Formation of the DISC in Response to Fas Activation. After ligation of Fas (CD95) by FasL (CD95L) this allows for the recruitment of FADD via homotypic interactions between DD. FADD will then facilitate recruitment of the effector molecules procaspase-8, -10 and c-FLIP through homotypic interactions of their DED.

The events immediately following Fas activation that allows for the formation of the DISC remain unclear but recent evidence provided by X-ray crystallography has begun to shed light on the issue. In response to ligand binding, Fas undergoes a conformational change altering its three dimensional structure[135,136]. This shape change

exposes the Fas cytoplasmic DD which subsequently allows for interactions with other DD containing proteins. The major scaffold protein responsible for the recruitment of effector molecules and stabilization of the DISC is the Fas-associated death domain (FADD)[130]. FADD is a cytosolic protein that possesses a DD near its C-terminal which is responsible for mediating its association with activated Fas. Near its N-terminal FADD possesses another protein-protein interaction domain capable of mediating homotypic interactions called a death effector domain (DED)[109]. FADD is responsible for the recruitment of DED containing effector molecules to the DISC, namely procaspase-8, procaspase-10 and c-FLIP[114].

Caspases comprise a family of cysteine proteases that are responsible for initiating and executing the apoptotic program. All caspases are initially translated in an enzymatically inactive, or pro-, form also known as a zymogen. In order for caspases to acquire enzymatic activity, they need to be catalytically processed. There are two generic categories of caspases: initiator and executioner caspases. Caspases-8, -9, and -10 are considered initiator caspases because these are the first caspases activated in response to various forms of pro-apoptotic stimuli. The initiator caspases are capable of auto-catalytically processing themselves and as such can positively regulate their own activity. Caspases-3, -6, and -7 are the major executioner caspases. These caspases are substrates for the initiator caspases which are responsible for processing them from their inactive form to their enzymatically active form. Once active, they recognize and cleave their corresponding substrates which cause the morphological changes observed in cells undergoing apoptosis.

Caspases-8 and -10 are the initiator caspases activated in response to Fas activation[137,138]. Procaspase-8 exists in two major isoforms, procaspase-8a and -8b, with both isoforms present in the DISC[139,140]. Procaspase-8 has limited substrate specificity and only enzymatically processes itself or c-FLIP[141]. Currently, the favored model for caspase-8 activation is the induced proximity model of initiator caspase activation[142]. The current evidence suggests that when procaspase-8 is recruited to FADD in response to Fas activation this allows for an accumulation of enzyme in relative close proximity[132,137]. FADD acts as a scaffold which allows for procaspase-8 molecules to form dimers. These procaspase-8 dimers are capable of catalytically modifying and activating other procaspase-8 dimers[142–144]. Conversion of procaspase-8 to caspase-8 causes a substrate switch allowing for enzymatic recognition of a more diverse set of substrates[141].

The conversion of procaspase-8 to the more enzymatically active caspase-8 occurs rapidly after receptor activation. Using co-immunoprecipitation and subsequent immunoblotting experiments, the active form of caspase-8 was detected at the DISC 30 seconds after receptor activation[140]. A steady increase in caspase-8 was detected at the DISC through 10 minutes with a drastic decrease in enzyme presence 20 minutes after receptor activation. Interestingly, the cytosolic pool of caspase-8 was detectible after 10 minutes and plateaued 20 minutes after receptor activation[140]. Once in the cytosol, active caspase-8 would be capable of activating executioner caspases leading to the fulfillment of the apoptotic program resulting in cell death.

Chapter 2: Effects of GBM-derived Exosomes on T cell viability

Introduction

Glioblastoma multiforme is the most common and aggressive primary brain tumor in adults. The average survival rate after diagnosis is only 15 months, despite an aggressive treatment regime consisting of surgical resection, chemo- and radio-therapy[6,9]. One of the confounding factors responsible for this dismal prognosis is the immune suppression that occurs both locally, in the tumor microenvironment, as well as systemically[41,145]. Typically, GBM patients are not immune suppressed prior to tumor development, and upon resection of the tumor some aspects of cellular immunity return to normal[39]. However, a generalized immune suppressive state is reinstated following tumor recurrence. Considering the location of the tumor, a vast majority of peripheral lymphocytes never come into direct contact with the tumor. Taken together, these observations highlight the role for a tumor specific soluble factor or factors responsible for these immune suppressive effects. While much research has gone into investigating possible mechanisms of immune suppression in GBM, a potential the role for tumor-derived exosomes remains poorly characterized.

Exosomes are small membrane bound vesicles of endocytic origin that contain mRNA, soluble proteins and transmembrane proteins[70]. Exosomes are released by a variety of cell types including cancer cells and activated immune cells[78,79,95,97,98]. Depending on their origin, exosomes can mediate a diverse array of physiologic functions. Exosomes isolated from tumor cells have been implicated in facilitating tumor progression, angiogenesis and immune suppression[96,98,99]. Specifically, exosomes isolated from melanoma, colorectal and prostate cancer cell lines contain the pro-apoptotic

molecule FasL and are capable of inducing apoptosis in activated lymphocytes[99–101].

While it is known that GBM cells release exosomes that are capable of transferring a

functional form of the oncogenic receptor EGFRvIII to other tumor cells[95,96], the effect of

GBM-derived exosomes on the immune system has not been satisfactorily described.

Utilizing co-culture systems and cell viability assays with Jurkat T cells GBM-

derived exosomes, we investigated whether the release of exosomes might be an immune

suppressive mechanism employed by glioblastoma multiforme. Our data demonstrate that

GBM-derived exosomes are capable of reducing T cell viability and therefore could be a

contributing factor to the observed systemic immune suppression observed in brain tumor

patients. These are novel findings in glioma biology.

Methods

Cell lines

The established glioblastoma cell lines, T98G, LN-229 and U-138MG, were

purchased from American Type Culture Collection (ATCC). GBM cells were maintained

in Essential Modified Eagle's Medium (EMEM) (Lonza) and supplemented with a final

concentration of 10% fetal bovine serum (FBS) (PAA Laboratories) and penicillin and

streptomycin (Lonza). The A3 T cell line (ATCC), which is a Jurkat T cell clone that is

hypersensitive to FasL-induced apoptosis, and maintained in RPMI-1640 (Lonza)

supplemented with 10% FBS (PAA Laboratories), 2mM sodium pyruvate (Lonza) and

penicillin and streptomycin (Lonza). All cells were cultured in a humidified incubator at

37°C with 5% CO_2.

Exosome Isolation

GBM cells were grown in 150cm^2 flasks until approximately 80% confluent, the culture supernatant was then aspirated and the cells were washed with sterile 1x PBS. After the cells were washed, the exosome isolation medium was added to the cells. The isolation media consisted of phenol red free DMEM (Lonza) supplemented with 2mM L-glutamine (Lonza) and penicillin and streptomycin (Lonza). After 30 hours the exosome collection medium was removed and the cells were maintained with normal culture medium. Exosome isolation was conducted by one of two methods: differential ultracentrifugation or centrifugal concentration.

For differential ultracentrifugation, the exosome collection medium was centrifuged at 500 x *g* for 10 minutes in order to remove dead cells. The supernatant was removed and centrifuged at 10,000 x *g* for 30 minutes in order to remove cellular debris and cell fragments. The resulting supernatant was ultracentrifuged at 100,000 x *g* for 90 minutes in order to pellet the exosomes. The supernatant was discarded and the exosome pellet was resuspended in 100μL of pre-chilled sterile 1x PBS. All centrifugation steps were performed at 4°C.

For exosomes collected using centrifugal concentration, the exosome collection medium was centrifuged at 500 x *g* for 10 minutes in order to pellet dead cells. Then the supernatant was removed and centrifuged at 10,000 x *g* for 30 minutes to remove cell fragments and cellular debris. The resulting supernatant was then applied to a 100,000 Dalton centrifugal filter (Millipore) and centrifuged at 3,220 x *g* for 20 minutes. All centrifugation steps were performed at 4°C using pre-chilled centrifuge tubes. Once the

sample was concentrated, sterile PBS was used to wash the filters. Final sample volume was around 300μL.

Cell Viability Assay

In order to determine the effect of GBM-derived exosomes on T cell viability, the Jurkat T cells were seeded in a 96-well plate at a final concentration of 5,000 cells/well. The cells were allowed to acclimate for 24 hours and were then treated with GBM-derived exosomes, 100ng/mL of rFasL (Enzo Life Sciences), or phosphate buffered saline (PBS). After 24 hours viability was assessed using the Cell TiterGlo viability assay (Promega) according to the manufacturer's directions.

Results

Jurkat T cells were co-cultured with exosomes isolated by differential ultracentrifugation for 24 hours, and following the incubation, a cell viability assay was performed. Exosomes isolated from T98G, U87, LN-229 (Figure 5a) and U-138 MG (Figure 5b) were all capable of significantly decreasing T cell viability when compared to the untreated control.

A

B

C
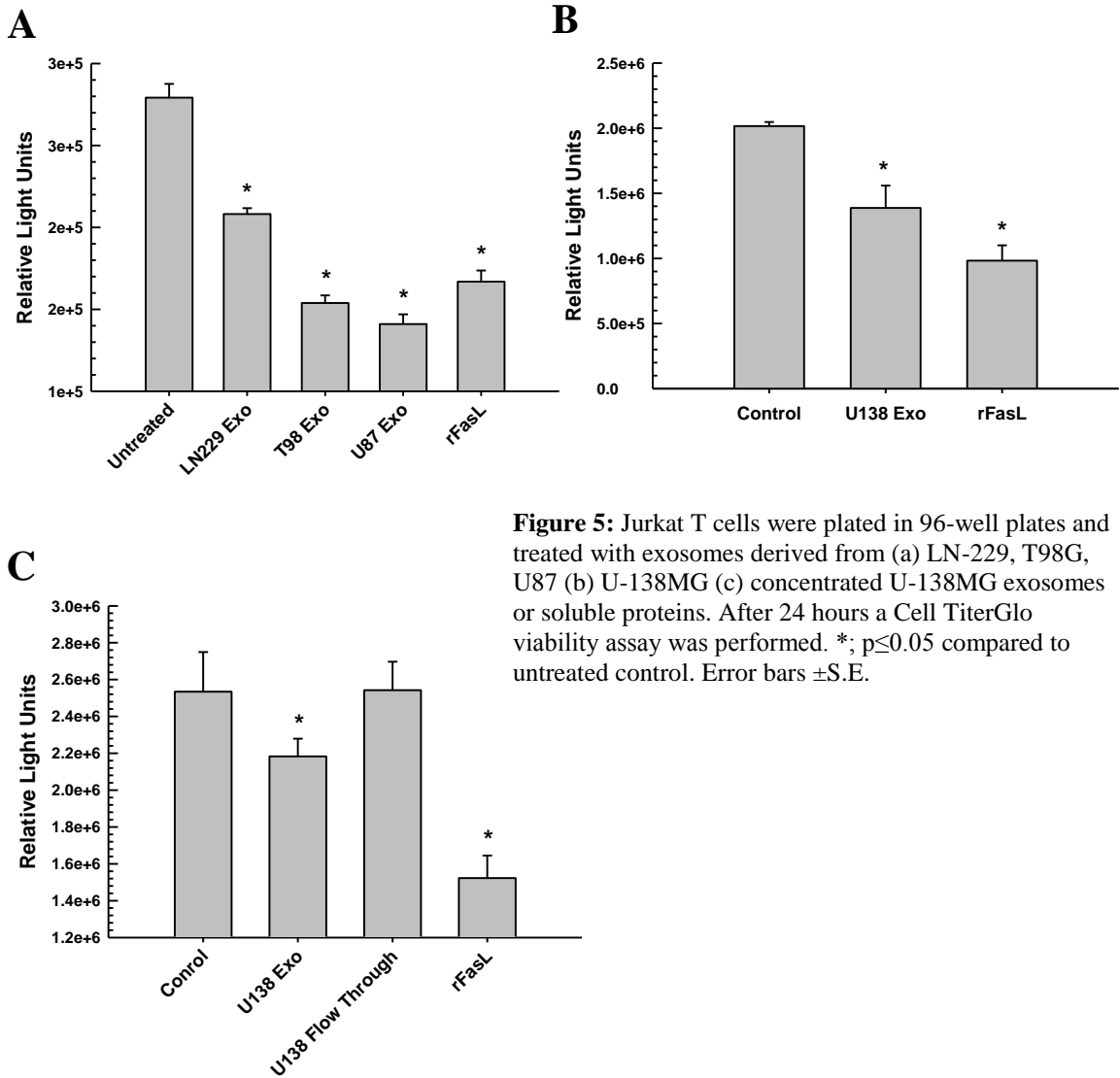
Figure 5: Jurkat T cells were plated in 96-well plates and treated with exosomes derived from (a) LN-229, T98G, U87 (b) U-138MG (c) concentrated U-138MG exosomes or soluble proteins. After 24 hours a Cell TiterGlo viability assay was performed. *; $p \leq 0.05$ compared to untreated control. Error bars ±S.E.

When Jurkat T cells were treated with the exosome-enriched fraction isolated using a 100kDa centrifugal filter from U-138 MG cells, the exosome fraction significantly decreased cell viability compared to the controls (Figure 5c). Importantly, when the Jurkat T cells were treated with the exosome-depleted centrifugal filter flow through, there was no change in cell viability compared to untreated control (Figure 5c).

Discussion

In order to better appreciate the effects GBM-derived exosomes could exert on the immune system, exosomes were isolated from several established brain tumor cell lines. Co-culture of GBM-derived exosomes with Jurkat T cells was sufficient to significantly decrease T cell viability (Figure 5a, 5b). However, due to the technique used to isolate the exosomes, the possibility of a contaminating soluble protein mediating these effects could not be ruled out. This confound was addressed by using 100kDa centrifugal filters to concentrate our exosome sample. This method specifically allowed for the separation of large macromolecular complexes and smaller secreted factors present in the culture medium. A decrease in cell viability was observed in T cells treated with the exosome fraction and not the soluble protein fraction (Figure 5c). Taken together, these data suggest that GBM-derived exosomes, and not some other secreted molecule, are responsible for the observed decrease in T cell viability.

We have demonstrated that GBM-derived exosomes are capable of reducing T cell viability compared to the untreated control. Mechanistically, cell death pathways may have been activated by exosome associated proteins such as FasL, but the activation of cascades that result in cell cycle arrest, such as CTLA-4 or TGF-β signaling, could not be eliminated. In order to determine if extrinsic apoptosis is activated by GBM-derived exosomes, the presence of FasL in the exosome samples was investigated.

Chapter 3: Characterization of GBM Cell Lines and Derived Exosomes

Introduction

Depending on the cell of origin, exosomes are capable of facilitating a broad range of physiologic functions. Exosomes isolated from dendritic cells are capable of

activating T cells and stimulating antitumor immune response leading to tumor clearance[146,147]. However, exosomes isolated from tumor cells are capable of mediating pro-tumor effects. *In vitro* experiments suggest that the horizontal transfer of exosome components between tumor cells could be responsible for mediating tumor progression.

Exosomes isolated from glioblastoma cells possess the oncogenic receptor EGFRvIII and can transfer the functional receptor between tumor cells[95,96]. The successful transfer of this receptor is sufficient to increase proliferation of the recipient tumor cells[95,96], as well as increase the expression of the anti-apoptotic protein Bcl_{XL}[95]. Exosomes isolated from gastric cancer cells are capable of activating the MAP kinase and Akt pathways in other cancer cells. In this manner, bulk tumor cells could be capable of stimulating proliferation and cell survival pathways in adjacent tumor cells via exosome release.

Tumor-derived exosomes may play a role in angiogenesis. Colorectal cancer-derived exosomes are enriched in mRNAs involved in the M phase of mitosis and are capable of increased endothelial cell proliferation[98]. Likewise, exosomes isolated from GBM cells are enriched in mRNA and proteins involved in the angiogenic process[96].

A role for tumor-derived exosomes has also been implicated in immune suppression. Exosomes isolated from a variety of cancer types contain the pro-apoptotic molecule FasL and are capable of inducing apoptosis in activated T cells[99–101]. Antagonistic antibodies to either FasL or its receptor, Fas, are sufficient to rescue T cells from apoptosis. These data suggest that exosome release may be an important mechanism of immune suppression. This form of immune suppression has not been reported for GBM.

Previous experiments demonstrated that GBM-derived exosomes are capable of decreasing T cell viability (Figure 5). These observations could be due to activation of pathways responsible for cell cycle arrest or, more likely, induction of extrinsic apoptosis. A major ligand of the death receptors responsible for induction of extrinsic apoptosis is FasL[112]. Using immunoblot techniques, we were able to demonstrate that FasL is expressed by established GBM cell lines; however, we but could not detect FasL in the exosome fraction.

Methods

Cell lines

The established glioblastoma cell lines, T98G, LN-229 and U-138MG, were purchased from American Type Culture Collection (ATCC). They were maintained in EMEM (Lonza) and supplemented with a final concentration of 10% FBS (PAA Laboratories) and penicillin and streptomycin (Lonza). The A3 T cell line is a Jurkat T cell clone that is hypersensitive to FasL induced apoptosis, was maintained in RPMI-1640 (Lonza) supplemented with 10% FBS (PAA Laboratories), 2mM sodium pyruvate (Lonza) and penicillin and streptomycin (Lonza). All cells were cultured in a humidified incubator at 37°C with 5% CO_2.

Exosome Isolation

GBM cells were grown in 150cm^2 flasks until approximately 80% confluent, the culture supernatant was aspirated, and the cells were washed with sterile 1x PBS. After the cells were washed, the exosome isolation medium was added to the cells. The isolation media was phenol red free DMEM (Lonza) supplemented with 2mM L-glutamine (Lonza) and penicillin and streptomycin (Lonza). After 30 hours, the exosome

collection medium was removed and the cells were maintained with normal culture

medium.

Exosomes were collected using centrifugal concentration. The exosome collection

medium was centrifuged at 500 x *g* for 10 minutes in order to pellet dead cells. The

supernatant was removed and centrifuged at 10,000 x *g* for 30 minutes to remove cell

fragments and cellular debris. The resulting supernatant was then applied to a 100,000

Dalton centrifugal filter (Millipore) and centrifuged at 3,220 x *g* for 20 minutes. All

centrifugation steps were performed at 4°C using pre-chilled centrifuge tubes. Once the

sample was concentrated sterile PBS was used to wash the filters. The final sample

volume was 300µL.

Reverse Transcriptase PCR

Total RNA was isolated from established GBM cell lines using IBI Total RNA

mini kit following the manufacturer's instructions. Total RNA was then converted into

cDNA using the Reverse Transcription System (Promega). Amplification of FasL or actin

from the first stand cDNA was performed by polymerase chain reaction using the

following primers:

| FasL Forward: |
| ATGTTTCAGCTCTTCCACCTACAGAAGGA |
| FasL Reverse: |
| CAGAGAGAGCTCCAGATATCGAA |
| Actin Forward: |
| AGTCCTGTGGCATCCACGAAACTA |
| Actin Reverse: |
| ACTCCTGCTTGCTGATCCACATCT |

The resulting PCR product was separated on a 1% agarose gel in 1x Tris Acetate EDTA buffer.

Western blot

Whole cell lysates were prepared by growing cells in culture conditions until they were 80% confluent. The culture medium was then aspirated and the cells were washed with an excess of 1x PBS. The cells were lysed using 1x SDS lysis buffer (20mM HEPES and 1% SDS) and genomic DNA was sheared using a syringe and a 12 gauge needle. Protein concentrations of the lysates were determined using the BCA assay (Thermo Scientific). Samples were resolved on pre-cast SDS-PAGE gels (BioRad) and transferred to nitrocellulose.

The nitrocellulose membranes were blocked for 30 minutes in a 5% non-fat milk solution in Tris buffered saline with Tween 20 (20mM Tris, 150mM NaCl, 0.1% Tween). After the membranes had been blocked, the nitrocellulose membranes were probed with primary antibodies, either anti-FasL or anti-CD9 (Abcam), for 1 hour. Then the nitrocellulose membranes were probed with HRP-conjugated anti-mouse secondary antibody for an hour. Bands were visualized using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific).

Results

As determined by RT-PCR, all three GBM cell lines investigated (T98G, U-138MG, LN-229) express the FasL transcript (Figure 6). To further verify the expression of FasL, Western blot analysis was used. As shown in Figure 7, all three cell lines expressed the FasL protein. However, neither FasL nor the exosomal marker CD9 was detected in GBM-derived exosomes (Figure 7).
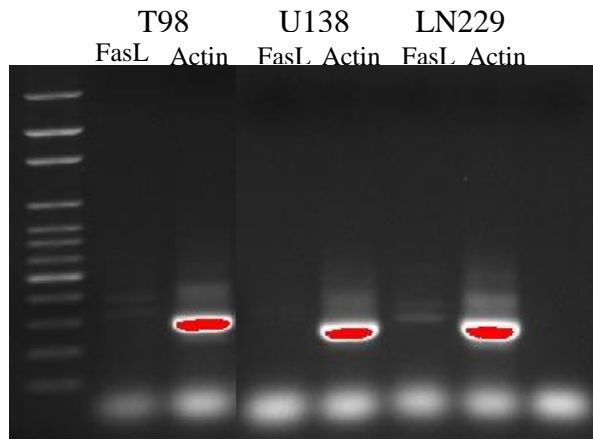
Figure 6: Total RNA was isolated from the indicated cell lines and was reverse transcribed to cDNA using random oligomers. The transcripts for FasL or Actin were specifically amplified using polymerase chain reaction and subsequently run on a 1% agarose gel using Tris Acetate EDTA running buffer.
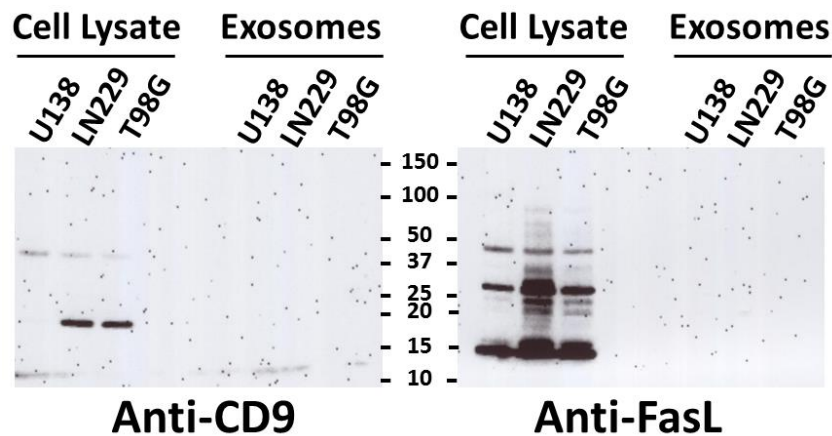
Figure 7: Whole cell lysates or exosome lysates were separated on a 4-15% polyacrylamide gel. Proteins were transferred to nitrocellulose membrane and probed for the exosomal marker CD9 or FasL. FasL is 35kDa and CD9 is 20kDa.

Discussion

FasL expression by established GBM cell lines was determined at the mRNA level as well as at the protein level (Figures 6 and 7). The bands corresponding to the amplified FasL transcript (500bp) in all three cell lines were detected after gel electrophoresis (Figure 6). In order to be confident that the cell lines expressed FasL,

Western blot was used to determine the presence of the protein. Consistent with the results from the RT-PCR experiments, FasL was detected in all cell lines investigated (Figure 7). However, the expression of FasL by the parent cells does not guarantee the presence of FasL in GBM-derived exosomes. Exosomes were isolated using a 100kDa centrifugal filter and subjected to Western blot for FasL and the exosomal marker CD9. Neither FasL nor CD9 was detected in the exosome fraction, despite both being found in the parent cell lines (Figure 7). These results do not rule out the presence of FasL in the exosome fraction. Because the exosomal marker CD9 was not detected, this could indicate that the protein concentration of the exosome fraction was below the detection limit for Western blots. Therefore, considering a signal for the exosomal marker CD9 was not detected, the lack of a FasL signal may only indicate that there was not a sufficient amount of the protein in the exosome fraction to produce a signal.

Chapter 4: Induction of Caspase Cascade by GBM-derived Exosomes

Introduction

A role for exosome mediated immune suppression has been implicated in a variety of tumor types including melanoma, colorectal and prostate cancer. These cancer types express the pro-apoptotic molecule FasL which binds to its receptor, Fas, and activates the extrinsic cell death pathway. Exosomes isolated from these cell types also contain FasL and are capable of inducing FasL-dependent apoptosis in activated T cells[99–101]. Studies show that established GBM cell lines, as well as freshly resected clinical samples, express FasL.

A recent study highlighted the importance of FasL expression in GBM in mediating immune suppression. Rat glioma cell lines (which normally express FasL)

were engineered to no longer express the protein and *in vitro* apoptosis assays indicated the FasL knockout cell lines induced significantly less T cell death compared to the wild type cell line[68]. Additionally, rats that were injected with the FasL knockout cells had a longer overall survival compared to rats who received the wild type cells[68]. When the tumors from the treated and control rats were examined for the presence of tumor infiltrating lymphocytes there were more lymphocytes, in the FasL knockout tumors than the wild type tumors[68]. The increased survival time of the FasL knockout tumors can be attributed to the elevated presence of lymphocytes in the tumor bed. This would indicate that FasL expression is a major component of the immune suppressive repertoire utilized by GBM to escape recognition and subsequent clearance by the immune system.

The ability of GBM to mediate immune suppression through the induction of lymphocyte apoptosis appears to be an important mechanism in immune suppression. However, a role for GBM-derived exosomes in immune suppression has not been investigated. In order to determine if GBM-derived exosomes are capable of activating the extrinsic apoptotic pathway, the activity of downstream effector molecules was investigated. In response to death receptor activation, a series of enzymatic reactions occur resulting in the activation of a class of proteins called caspases. Directly downstream of the death receptor is caspase-8. In response to death receptor activation caspase-8 is activated which activates caspase-3[112,140]. This caspase cascade results in apoptotic program and cell death. In order to determine the ability of GBM-derived exosomes to induced T cell apoptosis the activity of downstream caspases was investigated.

Methods

Cell lines

The established glioblastoma cell lines, T98G, LN-229 and U-138MG, were

purchased from ATCC. They were maintained in EMEM (Lonza) and supplemented with

a final concentration of 10% FBS (PAA Laboratories) and penicillin and streptomycin

(Lonza). The A3 T cell line is a Jurkat T cell clone that is hypersensitive to FasL induced

apoptosis was maintained in RPMI-1640 (Lonza) supplemented with 10% FBS (PAA

Laboratories), 2mM sodium pyruvate (Lonza) and penicillin and streptomycin (Lonza).

All cells were cultured in a humidified incubator at 37°C with 5% CO_2.

Exosome Isolation

GBM cells were grown in 150cm^2 flasks until approximately 80% confluent, the

culture supernatant was aspirated and the cells were washed with sterile 1x PBS. After

the cells were washed the exosome isolation medium was added to the cells. The

isolation media was phenol red free DMEM (Lonza) supplemented with 2mM L-

glutamine (Lonza) and penicillin and streptomycin (Lonza). After 30 hours the exosome

collection medium was removed and the cells were maintained with normal culture

medium.

Exosomes were collected using centrifugal concentration. The exosome collection

medium was centrifuged at 500 x *g* for 10 minutes in order to pellet dead cells. Then the

supernatant was removed and centrifuged at 10,000 x *g* for 30 minutes to remove cell

fragments and cellular debris. The resulting supernatant was then applied to a 100,000

Dalton centrifugal filter (Millipore) and centrifuged at 3,220 x *g* for 20 minutes. All

centrifugation steps were performed at 4°C using pre-chilled centrifuge tubes. Once the

sample was concentrated sterile PBS was used to wash the filters. Final sample volume was around 300μL.

Caspase Activity Assay

Jurkat T cells were seeded in a 96-well plate at a final concentration of 5,000 cells/well. The cells were allowed to acclimate for 24 hours and were treated with GBM-derived exosomes, 100ng/mL of rFasL (Enzo Life Sciences) or phosphate buffered saline (PBS). Activation of a caspase cascade in response to exosome or rFasL treatment was determined using Caspase-8 Glo or Caspase-3/7 Glo (Promega), per the manufacturer's instructions. Neutralizing assays were conducted using the Fas antagonist monoclonal antibody ZB4 (Millipore). Jurkat T cells were pretreated with 500ng/mL of ZB4 for 1 hour before the addition of either GBM-derived exosomes or rFasL.

Results

Jurkat T cells were treated with increasing concentrations of rFasL to determine sensitivity to FasL-mediated caspase-8 activation. rFasL was able to increase caspase-8 activity in Jurkat T cells at all concentrations and time points (Figure 8). Pre-treatment of Jurkat T cells with the Fas neutralizing antibody, ZB4, prior to addition of rFasL blocked caspase activation (Figure 9). Treatment with the antibody alone did not effect T cell viability (Figure 9).
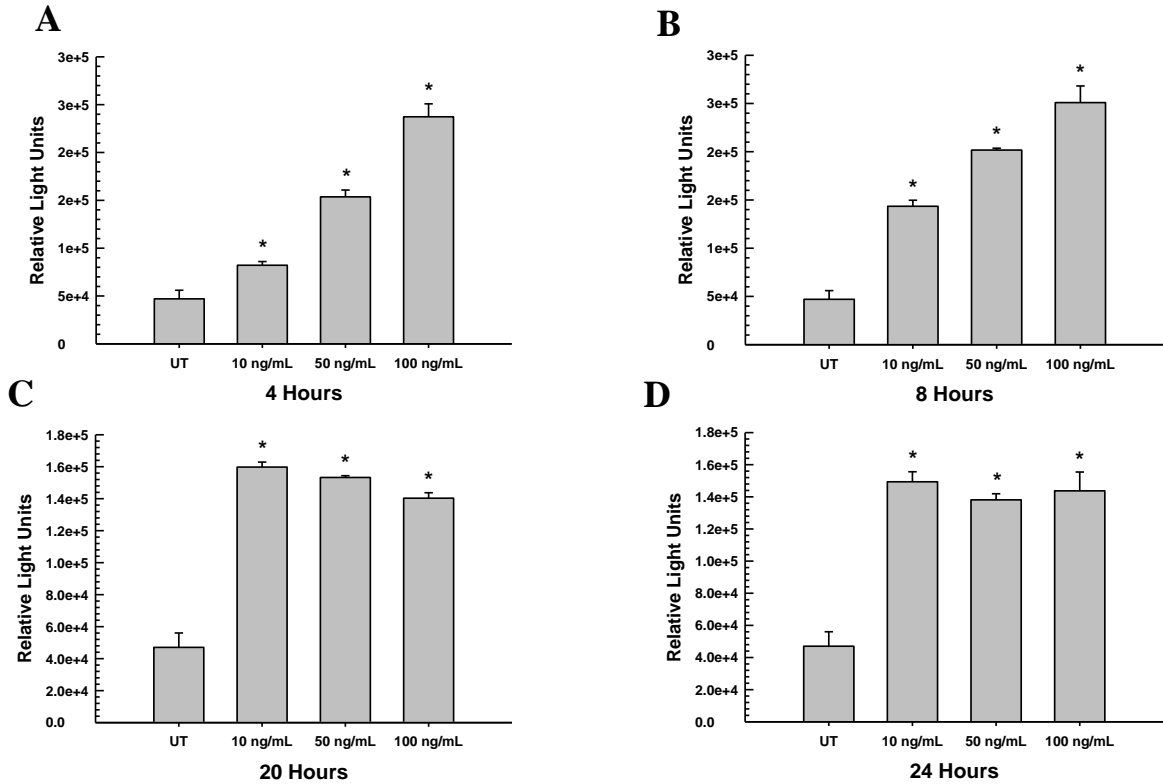
Figure 8: Jurkat T cells were plated in 96-well plates at a concentration of 50,000 cells/mL and were treated with either 10ng/mL, 50ng/mL or 100ng/mL of rFasL. Caspase-8 activity in response to rFasL treatment was assayed (a) 4 hours (b) 8 hours (c) 20 hours or (d) 24 hours post-treatment using Caspase-8Glo. *; $p \leq 0.05$ compared to UT. Error bars ±S.E.
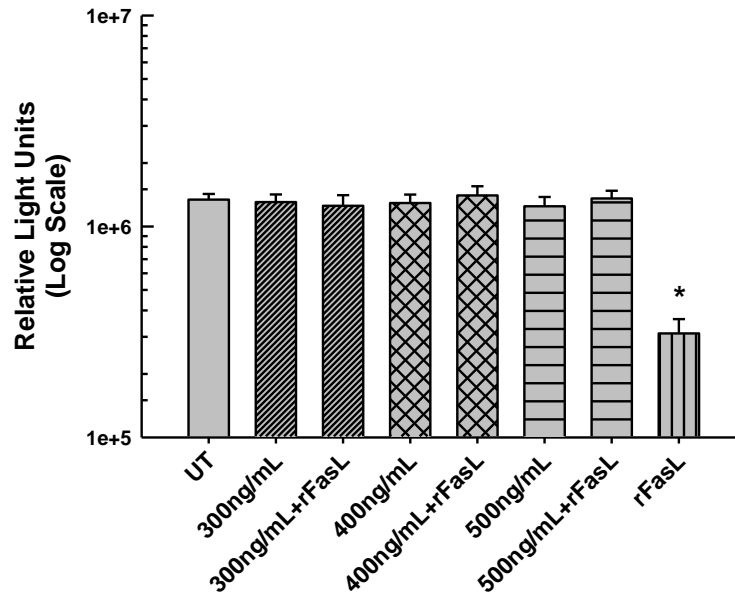
Figure 9: Jurkat T cells were plated in a 96-well plate at a concentration of 50,000 cells/mL and were treated with the Fas neutralizing antibody, ZB4. Jurkat T cells were treated with the indicated concentration of ZB4 alone in combination with 100ng/mL of rFasL. After 24 hours a Cell TiterGlo viability assay was performed. *; $p \leq 0.05$ compared to UT. Error bars ±S.E.

Co-culturing Jurkat T cells with GBM-derived exosomes caused a significant increase in caspase-8 activity (Figure 10). Exosomes derived from T98G cells activated caspasae-8 significantly more than LN-229- or U-138MG-derived exosomes. T98G-derived exosomes were also capable of increasing caspase-3 activity (Figure 11).

Figure 10: Jurkat T cells were plated in 96-well plates at a concentration of 50,000 cells/mL and were treated with exosomes derived from T98G, LN-229, U-138MG or with rFasL. After 24 hours caspase-8 activity was determined using Caspase-8Glo. *; $p \leq 0.05$ compared to UT. Error bars ±S.E.

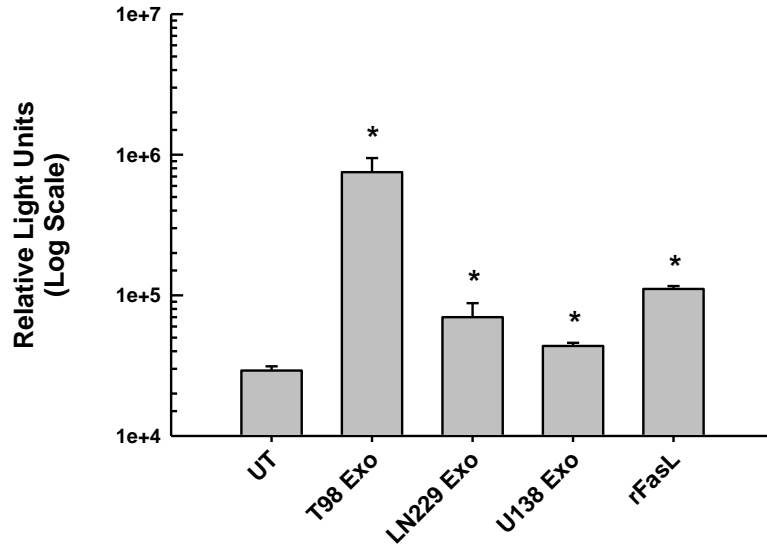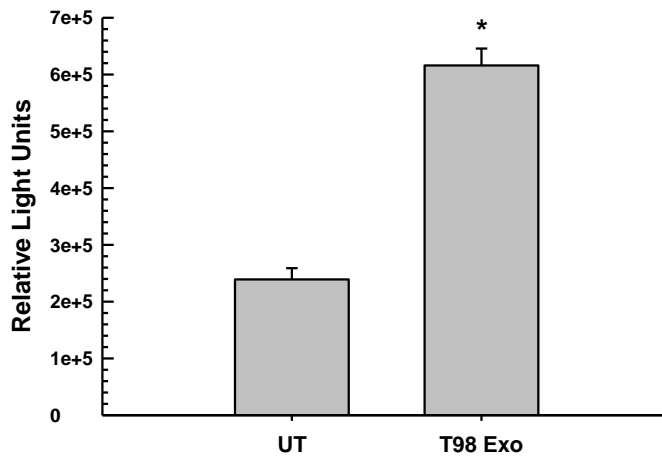**Figure 11:** Jurkat T cells were plated in 96-well plates at a concentration of 50,000 cells/mL and treated with T98G-derived exosomes. After 4 hours caspase-3 activity was determined using Caspase-3/7Glo. *; $p \leq 0.05$ compared to UT. Error bars ±S.E.

Pre-treatment of Jurkat T cells with ZB4 prior to co-culture with GBM-derived

exosomes produced variable results. Exosomes isolated from all three GBM cell lines

were capable of increasing caspase-8 activity. However, the combination of ZB4 and U-

138MG- and LN-229-derived exosomes significantly increased caspase-8 activation

compared to exosome treatment alone (Figure 12). There was no difference in caspase-8

activity in the presence or absence of ZB4 when Jurkat T cells treated with T98G-derived
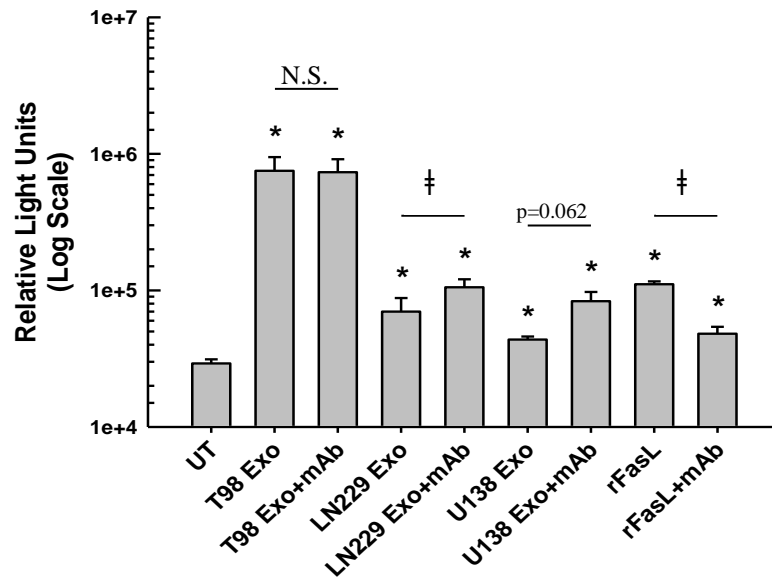
exosomes (Figure 12).

Figure 12: Jurkat T cells were plated in 96-well plates at a concentration of 5,000 cells/well. Groups labeled mAb were pretreated with a Fas neutralizing antibody before being co-cultured with GBM-derived exosomes. After 24 hours caspase-8 activity was determined using Caspase-8Glo. * indicates significance (p≤0.05) with the untreated control. ‡ indicates significance (p≤0.05) between the indicated treatment groups. Error bars ±S.E.

Jurkat T cells treated with ZB4 and exosomes had significantly decreased cell

viability compared to cells treated with exosomes alone (Figure 13). Consistent with the

observed changes in caspase-8 activity these differences were only observed in Jurkat T

cells treated with U-138MG- and LN-229-derived exosomes. There was no difference in

viability in T cells treated with T98G-derived exosomes (Figure 13).
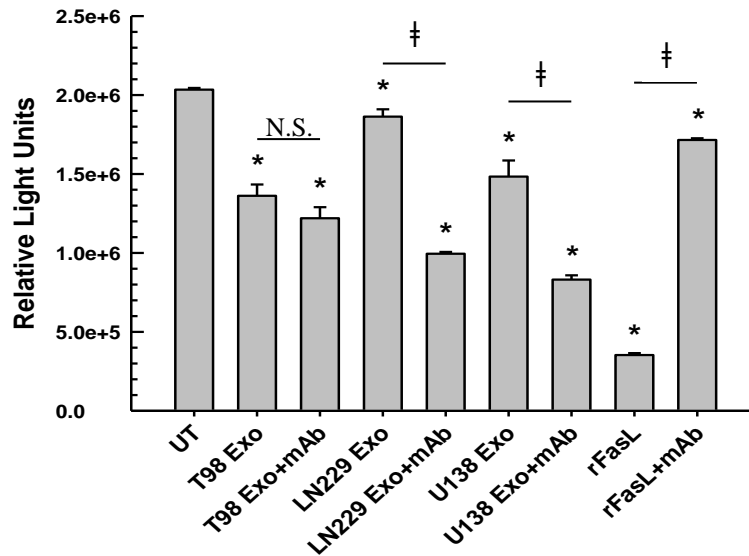
Figure 13: Jurkat T cells were plated in 96-well plates. Groups labeled mAb were pre-treated with ZB4 before being co-cultured with GBM-derived exosomes. After 24 hours a Cell TiterGlo viability assay was performed. *; indicates significance (p≤0.05) with untreated. ‡; indicates significance between treatments. Error bars ±S.E.

Discussion

The ability of GBM-derived exosomes to induce T cell apoptosis via a FasL-dependent mechanism was investigated in response to reports of exosomes from other cancer types being able to mediate these effects. First, the sensitivity of Jurkat T cells to FasL-mediated apoptosis was assessed. Jurkat T cells were treated with increasing concentrations of rFasL and caspase-8 activity was determined at 4, 8, 20 or 24 hours post-treatment. Caspase-8 is immediately downstream of Fas activation, therefore assaying its activity is a good indicator for the signaling status of the receptor. Caspase-8 activity increased in response to all concentrations of rFasL used (Figure 8). A dose-dependent increase of caspasase-8 activity was observed at 4 and 8 hours post-treatment (Figure 8a, 8b). Increased concentrations of rFasL should result in greater receptor activation, and thus an increase in caspase-8 activity. After 20 hours and through 24

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hours post-treatment caspase-8 activity remained elevated but had reached a steady state (Figure 8c, 8d).

In order to determine the specificity of these effects to the Fas/FasL pathway the Fas neutralizing antibody, ZB4, was used. Jurkat T cells were pre-treated with increasing concentrations of neutralizing antibody for one hour before treatment with rFasL. Pre-treatment of cells with the Fas neutralizing antibody was capable of rescuing T cell viability at all concentrations used (Figure 9). Treatment of Jurkat T cells with only Fas neutralizing antibody had no effect on viability at any of the concentrations used (Figure 9). Taken together, these data indicate that treatment of Jurkat T cells with rFasL is sufficient for the induction of caspase-8 activity and capable of reducing T cell viability.

These experiments were replicated using GBM-derived exosomes. Caspase-8 activity increased in Jurkat T cells that were treated with exosomes isolated from LN-229, U-138MG or T98G (Figure 10). However, T98G-derived exosomes had the greatest effect on enzyme activity. Therefore, T98G-derived exosomes were used to determine if GBM-derived exosomes could activate a caspase cascade indicative of FasL signaling. Not only were T98G-derived exosomes capable of inducing caspase-8 activity, but this resulted in a significant increase of caspase-3 activity as well (Figure 11). These data indicate that GBM-derived exosomes are capable of activating a caspase cascade indicative of FasL signaling.

Next, T cells were treated with ZB4 before the addition of GBM-derived exosomes. Jurkat T cells that were treated with both the Fas neutralizing antibody and exosomes isolated from LN-229 or U-138MG had significantly more caspase-8 activity than Jurkat T cells treated with just GBM-derived exosomes (Figure 12). There was no

difference in caspase-8 activity in T cells that were treated with T98G-derived exosomes in the presence or absence of ZB4 (Figure 12). Consistent with these observations Jurkat T cells that were treated with LN-229- or U-138MG-derived exosomes and ZB4 had decreased cell viability compared to Jurkat T cells that were only treated with GBM-derived exosomes (Figure 13). There was no difference in cell viability with T cells treated with T98G-derived exosomes (Figure 13).

Given the contradictory nature of the neutralizing antibody data it is feasible that the antibody facilitates Fas receptor cross-linking and subsequent receptor activation. This would lead to increased caspase-8 signaling and results in decreased T cell viability. However, given results of previous experiments, it is clear that ZB4 is capable of neutralizing the apoptotic effect of rFasL (Figure 9), and treatment of Jurkat T cells with ZB4 alone does not have an effect on T cell viability (Figure 9). The effects observed from combined treatment of Jurkat T cells with GBM-derived exosomes and ZB4 are specific to that treatment condition.

One possible explanation for these contradictory results could be that GBM-derived exosomes could contain an immunoglobulin receptor. When the Jurkat T cells were pre-treated with the Fas neutralizing antibody, this allowed for specific interactions between the antigen binding domain of the antibody to bind to its epitope on the Fas receptor. This would leave the F_c region of the antibody exposed. In the presence of an exosomal associated immunoglobulin receptor, the F_c regions of multiple antibodies could be bound. This could potentially lead to cross-linking of Fas receptor subunits and induce signaling in the absence of ligand binding. This possible mode of action is currently being investigated.

Chapter 5: Conclusions

Glioblastoma multiforme is a devastating disease with an average survival rate of 15 months after diagnosis[6]. Novel treatment strategies are desperately needed in the fight against brain tumors. Recent efforts have investigated the effectiveness of immune based therapies in the treatment of malignant gliomas[148]. Pre-clinical studies as well as phase I/II clinical trials have reported putative successes using immunotherapies targeting GBM[11,20–22,24,25,148]. However, to date, there are no FDA approved immunotherapies for the treatment of GBM. A potential confounding factor to the application of immunotherapies in GBM could be the degree of systemic immune suppression afforded by the tumor. If a patient's immune system is predisposed to be suppressed or hyporesponsive due to tumor specific factors, therapeutic intervention might not be sufficient to elicit a robust antitumor immune response. The diverse repertoire of immunosuppressive factors employed by GBM needs to be antagonized in order to increase the efficacy of immunotherapies. The most successful immunotherapeutic regime will simultaneously target the tumor itself as well as factors produced by the tumor responsible for immune suppression.

The immune system of GBM patients is severely compromised. Peripheral blood lymphocytes from GBM patients are hyporesponsive, and there is an overall decrease in the number of circulating lymphocytes[145]. These deficiencies are restored after tumor resection but quickly return with tumor recurrence[39]. This indicates that the factors responsible for immune suppression are tumor specific and since most peripheral lymphocytes never come into direct contact with the tumor the factors mediating immune suppression must be soluble. These molecules have the ability to diffuse away from the

tumor bed, enter into circulation and exert their immunomodulatory effects on distant populations of immune cells. GBM-derived exosomes are an attractive candidate that fulfills both of these proposed criteria for immune suppression. Considerable research has gone into investigating immunosuppressive mechanisms employed by GBM [41,145] but the role of GBM-derived exosomes has not been investigated to date.

Exosomes are small vesicles (50-100nm of endocytic origins that contain mRNA, miRNA and proteins. Exosomes are released by a variety of cell types, including tumor cells, and exosomes can facilitate diverse physiologic processes such as antigen presentation[78], angiogenesis[98] and tumor progression[95]. A role for exosomes in immune suppression for colorectal, prostate cancer and melanoma has been proposed. FasL is present in exosomes isolated from those cancer types and will activate extrinsic apoptosis when co-cultured with T cells[99–101]. Clinical samples, as well as established GBM cell lines, express FasL[68] but the presence of FasL in GBM-derived exosomes and its functional significance has never been investigated. We have shown that GBM-derived exosomes are capable of inducing apoptosis in Jurkat T cells. This is a novel finding in glioma biology.

In the present study we have shown that the established GBM cell lines LN-229, U-138MG and T98G all express the pro-apoptotic molecule FasL. This was shown by reverse transcriptase PCR and subsequent agarose electrophoresis (Figure 6) and confirmed with immunoblotting techniques (Figure 7). Expression of FasL by the parent cells does not necessarily mean that it will be present in the exosomes. Western blot analysis of GBM-derived exosomes was unable to detect either FasL or the exosomal marker CD9. The inability to detecte CD9 in our sample indicates that the protein

concentration was most likely below the detection level for Western blot analysis. The presence or absence of FasL in our samples cannot be determined from our data.

Functional assays were performed to determine the effect of GBM-derived exosomes on Jurkat T cells. The co-culture of Jurkat T cells with exosomes isolated from LN-229, T98G, U87 and U-138MG cell lines was sufficient to decrease T cell viability when compared to the untreated control (Figure 5a, b). However, membrane bound FasL can be enzymatically cleaved into a soluble isoform[149] which has been reported to be found in cyst fluid isolated from malignant gliomas[150]. In order to determine whether exosomes or a contaminating soluble protein were responsible for the decreased T cell viability, exosomes were isolated using 100kDa centrifugal filters. Components of the cell culture medium smaller than 100kDa should pass through the filter while components larger than 100kDa are retained. Only the exosome-enriched fraction was capable of decreasing T cell viability (Figure 5c). When Jurkat T cells were treated with the fraction containing secreted components <100kDa, there was no difference in T cell viability when compared to the untreated control (Figure 5c).

Taken together, these data indicate that all three GBM cell lines express FasL and exosomes isolated from those cell lines, not a secreted protein, are capable of decreasing T cell viability in co-culture assays. However, the molecular cargo of GBM-derived exosomes is relatively unknown. The exosomes could be activating cell cycle arrest pathways in T cell like TGF-β or CTLA-4 signaling. In order to determine if GBM-derived exosomes are capable of activating the FasL/Fas signaling pathway, the activity of downstream signaling molecules of Fas activation were investigated.

Shortly after FasL binds to Fas the inactive procaspase-8 is converted into the active form of the enzyme, caspase-8[140]. Therefore, assaying for caspase-8 activity is a good indicator of the signaling status of Fas. Caspase-8 activity is increased in Jurkat T cells that were co-cultured with GBM-derived exosomes (Figure 10). Specifically, T98G-derived exosomes had the greatest increase caspase-8 activity. After caspase-8 is activated in response to Fas signaling it in turn cleaves the inactive procaspase-3 which converts it into active caspase-3. Caspase-3 is referred to as an executioner caspase and is responsible for the fulfillment of the apoptotic program. Caspase-3 activity is increased in T cells co-cultured with T98G-derived exosomes (Figure 11). These data indicate that GBM-derived exosomes are sufficient to stimulate a caspase cascade indicative of FasL-induced apoptosis in T cells. In order to show that this signaling cascade was activated by FasL a series of experiments utilizing a Fas neutralizing antibody were performed.

Contradictorily, caspase-8 activity was increased in Jurkat T cells that had been treated with both ZB4 and exosomes derived from LN-229 or U-138MG when compared to T cells that had been only treated with LN-229- or U-138MG-derived exosomes (Figure 12). There was no significant difference in caspase-8 activity in Jurkat T cells treated with T98G-derived exosomes in the presence or absence of ZB4 (Figure 12). Caspase-8 activity inversely correlated with T cell viability in response to exosome treatment. Jurkat T cells that had been treated with both ZB4 and exosomes from LN-229 or U-138MG had reduced viability as compared to T cells treated with exosomes alone (Figure 13). There was no statistical difference in T cell viability when co-cultured with T98G-derived exosomes in the presence or absence of ZB4 (Figure 13).

To summarize, when Jurkat T cells are pre-treated with the Fas neutralizing antibody ZB4 then subsequently co-cultured with exosomes derived from LN-299 or U-138MG, they experienced more caspase-8 activity than in T cells treated with exosomes alone. The combination treatment also significantly decreases T cell viability when compared to T cells that were treated with only exosomes. However, there is no significant difference in caspase-8 activity or T cell viability in the presence or absence of ZB4 when T cells are treated with T98G-derived exosomes.

The Fas neutralizing is capable of blocking rFasL-induced apoptosis (Figure 9) and treatment of Jurkat T cells with ZB4 alone does not have an effect of T cell viability (Figure 9). Therefore, something about treating the Jurkat T cells with ZB4 and the exosomes is responsible for these contradictory results. One possibility is that following pre-treatment with ZB4 the antigen binding domain of the antibody binds to its epitope on Fas. This would normally be sufficient to disrupt the interactions between Fas and FasL which would inhibit the ability of Fas to induce apoptosis. However, if the GBM-derived exosomes contain an immunoglobulin receptor it is possible that they bind to the F_c regions of multiple ZB4 bound Fas receptors. This could lead to receptor cross-linking and subsequent activation. This would explain the significant increase in caspase-8 activity in T cells treated with both ZB4 and LN-229- or U-138MG-derived exosomes. It is possible that T98G cells do not express the immunoglobulin receptor or it could be absent from T98G-derived exosomes. This would explain the lack of change in either caspase-8 activity or T cell viability upon co-culture with T98G-derived exosomes in the presence or absence of ZB4.

This hypothesis could be tested by performing a papain digestion on the ZB4 antibody. Papain is a protease that is able to cleave the F_c region from IgG molecules. This would separate the F_c region from the antigen binding domains of the antibody. The ability of an immunoglobulin receptor to crosslinking antibody bound Fas is dependent on the F_c region. Therefore, if these experiments were repeated using only the antigen binding domain of ZB4 as the neutralizing agent it could be determined if the apoptotic effects of GBM-derived exosomes were augmented via this indirect mechanism.

Regardless of mechanism our data strongly indicates that GBM-derived exosomes are capable of inducing apoptosis in Jurkat T cells. Co-culturing Jurkat T cells with GBM-derived exosomes is sufficient for induction of apoptosis. This is a novel finding in glioma biology and indicates a role for exosome release in immune suppression. The development of therapies aimed at antagonizing the biogenesis and secretion of exosomes could be a useful adjuvant to current treatments.

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