Glioblastomas Induce T-Lymphocyte Death by Two Distinct Pathways Involving Gangliosides and CD70

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Abstract
Here we report that glioblastoma multiforme (GBM) mediates immunosuppression by promoting T-cell death via tumor-associated CD70 and gangliosides that act through receptor-dependent and receptor-independent pathways, respectively. GBM lines cocultured with T cells induced lymphocyte death. The GBM lines were characterized for their expression of CD70, Fas ligand (FasL), and tumor necrosis factor-α (TNF-α), and the possible participation of those molecules in T-cell killing was assessed by doing GBM/T cell cocultures in the presence of anti-CD70 antibodies, Fas fusion proteins, or anti-TNF-α antibodies. CD70 but not TNF-α or FasL is responsible for initiating T-cell death via the receptor-dependent pathway. Of the four GBM cell lines that induced T-cell death, three highly expressed CD70. Two nonapoptogenic GBM lines (CCF3 and U138), on the other hand, had only minimally detectable CD70 expression. Blocking experiments with the anti-CD70 antibody confirmed that elevated CD70 levels were involved in the apoptogenicity of the three GBM lines expressing that molecule. Gangliosides were found to participate in the induction of T-cell apoptosis, because the glucosylceramide synthase inhibitor (PPPP) significantly reduced the abilities of all four apoptogenic lines to kill the lymphocytes. High-performance liquid chromatography (HPLC) and mass spectrometry revealed that GM2, GM2-like gangliosides, and GD1a were synthesized in abundance by all four apoptogenic GBM lines but not by the two GBMs lacking activity. Furthermore, gangliosides isolated from GBM lines as well as HPLC fractions containing GM2 and GD1a were directly apoptogenic for T cells. Our results indicate that CD70 and gangliosides are both products synthesized by GBMs that may be key mediators of T-cell apoptosis and likely contribute to the T-cell dysfunction observed within the tumor microenvironment. (Cancer Res 2005; 65(12): 5428-38)

Introduction
Depressed T-cell function, resulting in ineffective antitumor immune response, is common among cancer patients (1). The tumor microenvironment has a deleterious effect on tumor-infiltrating T cells, and there is evidence that the immunosuppressive effects extend into the periphery as well (1). Even glioblastoma multiforme (GBM) that develops and remains usually within the brain can induce local and systemic host immunosuppression (2, 3). This impairment of the immune system, specifically when it affects T lymphocytes, can occur through mechanisms that increase the sensitivity of these cells to apoptosis.

Studies on the mechanisms by which tumor cells induce T-cell apoptosis have shown a role for tumor-associated Fas ligand (FasL) and other tumor necrosis factor (TNF)-related ligands that activate the receptor-dependent apoptotic pathway (4, 5). However, similar immune dysfunction can also be mediated by tumor cell culture supernatants, which when added to normal activated human T cells, also induce T-cell death (6). Likewise, tumor cyst fluid and cerebrospinal fluid from patients with gliomas suppressed normal lymphocyte activity (7). This is reminiscent of the finding that isolated, peripheral blood T cells from glioma patients are apoptotic (6), suggesting that gliomas secrete soluble products capable of distant immunosuppression. Given that GBM overexpress various gangliosides (8–10) with known immunosuppressive and apoptotic activities (11, 12), it is possible that these gangliosides are also involved in the observed apoptosis of T cells.

We defined the mechanisms of GBM-induced T-cell death in coculture experiments using multiple cell lines. GBM-induced apoptosis of T cells is most pronounced in activated T cells suggesting that the activation status of lymphocytes is an important determinant of sensitivity to tumor-mediated apoptosis. T-cell death induced by some GBM lines is partially receptor dependent, involving CD70 expression by the tumor cells. CD70, the ligand for CD27, seems the predominant receptor-dependent pathway involved in T-cell death by GBM lines. The addition of anti-CD70 antibody to the T-cell tumor cultures partially blocked T-cell death, whereas neither the Fas fusion protein nor anti-TNF-α antibody had any significant protective effect. Furthermore, death induced by both receptor-dependent GBM lines (CCF52, U87, D54, and U251) and the receptor-independent line (CCF4) involved the participation of tumor associated gangliosides. Blocking glycosphingolipid production with the glucosylceramide synthase inhibitor PPPP partially protected T cells from GBM-mediated apoptosis. Additionally, gangliosides isolated from the GBM lines independently induced apoptosis of T lymphocytes. Our results also indicate that some GBM tumor lines may induce T-cell apoptosis via a cooperative interaction between CD70 and gangliosides, because greater protection was observed when PPPP and anti-CD70 antibody were given in combination.

Materials and Methods
Reagents. The inhibitor of glucosylceramide synthase, 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPPP), was purchased from Matreya, Inc. (Pleasant Gap, PA). Monoclonal anti-CD3 (OKT3, Ortho Biotech, Raritan, NJ) and anti-CD28 antibody (Becton Dickinson Immunocytometry Systems, San Jose, CA) were used for the stimulation of T lymphocytes. Human recombinant interleukin-2 (200 units/ml; IL-2, [Proleukin], CHIRON Corp., Emeryville, CA) was used to maintain the viability of activated T cells. Mouse monoclonal anti-human CD70 and

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BU69 (Ancell, Bayport, MN) was used at a dilution of 10 μg/mL for blocking CD70 ligand. The isotype control for the CD70 antibody was purified mouse immunoglobulin G (BD Pharmingen, San Jose, CA). Human FasFc chimeric fusion protein (BD Pharmingen) was used at 7.5 μg/mL (optimal concentration). Mouse P-Selectin-immunoglobulin G fusion protein (BD Pharmingen) was used as the control at 7.5 μg/mL. Surface expression of FasL was detected using anti-FasL antibody from BD Pharmingen. Positive controls used for the efficiency of FasFc to block apoptosis included anti-Fas activating antibody (Upstate, Charlotteville, WA) and supernatants from ecotropic retroviral packing cells (GP+E 86) carrying human FasL-expressing retroviral particles (13).

Established cell lines and tissue culture conditions. The well characterized, long-term GBM cell lines: U87, U138, U251, and D54 were obtained courtesy of Dr. Vogelbaum (Cleveland Clinic Foundation, Cleveland, OH). These lines were maintained in DMEM (Bio Whittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 2 mmol/L L-glutamine, 50 μg/mL gentamicin. The Jurkat leukemic T-cell line (American Type Culture Collection, Rockville, MD) was maintained in complete RPMI 1640 (Bio Whitaker) supplemented as described above. All the cell lines were found free of Mycoplasma (ELISA, Roche Technologies).

Primary cell cultures. GBM specimens were obtained from our Brain Tumor Registry after being reviewed by a pathologist. Classification of tumor type and grade was made in accordance with the WHO histologic typing. Single-cell suspension of GBM cells were obtained following digestion as previously described (14). They were then seeded in flasks and subcultured for two to four passages before being verified to be GBM cells (14). Three short-term cultures (CFF3, CCF4, and CCF52) were obtained from three different patients.

Expression of CD70, Fas ligand, and tumor necrosis factor-α by glioblastoma multiforme lines. GBM lines were stained with anti-human CD70, anti-human FasL (BD Pharmingen) or immunoglobulin G isotype control for 60 minutes at 4°C. The cells were then incubated with a PE-conjugated goat anti-mouse immunoglobulin G antibody for 30 minutes at 4°C. The cells were fixed in 1% paraformaldehyde. At least 10,000 events were acquired on a FACSscan (BD Biosciences, San Jose, CA) with a 488-nm argon laser. Flow cytometric analysis was done using CellQuest v3.3 software (BD Biosciences). The GBM lines supernatants were harvested and assessed for TNF-α protein using an ELISA kit (Pierce Biotechnology, Rockford, IL). Total RNA was extracted from GBM and reverse transcription-PCR was done using 5.0 μg RNA from each line. Reverse Transcription-PCR Reagents (Applied Biosystems, Foster City, CA) and cycled on a Perkin-Elmer Cetus DNA thermal cycler. cDNA (2.0 μL) was used in the PCR reaction using SYBR green PCR core reagents (Applied Biosystems) with primers for TNF-α (forward primer 5'-CTTCTCCCTCTTTGATCTGGC-3' and reverse primer 5'-GATGATCTGCTGTCGCTGG-3'); in addition, forward primer 5'-CTCTCTTCCAGAAGGCCC-3' and reverse primer 5'-CCAGTGTAGCTACGACTGG-3'). The control gene was β-GUS (forward primer: 5'-CTTCTCAGGAGCCCTG-3', 3'-Gus reverse primer: 5'-ACTGGCCTTCTGGTGAGCCCG-3'). The cycle threshold (cT) values were used to calculate the fold differences of cytokine expression relative to the expression of the housekeeping gene, β-GUS.

T-cell isolation. T cells are isolated from peripheral blood first with a ficoll-paque gradient (Amersham, Upppsala, Sweden) followed by negative selection using StemSep antibody cocktail (StemCell Technologies, Vancouver, British Columbia).

Stimulation of T cells with cross-linked anti-CD3 antibody (OKT3) plus anti-CD28 antibody was done as previously described (15). The T cells were expanded for 2 to 3 weeks in the presence of 200 units/mL IL-2 before use.

Ganglioside isolation and profiling by high-performance liquid chromatography. A total lipid extraction of GBM cells was done using chloroform/methanol, and gangliosides were isolated from the lipids by a diisopropyl ether/1-butanol partition and subsequent Sephadex G-25 gel filtration step (16).

The water-soluble gangliosides were further fractionated by high-performance liquid chromatography (HPLC) using a Beckmann-Coulter HPLC system. A normal phase Varian Microsorb-NH2 column (25 cm, 100 Å) was used as the stationary phase. Ganglioside separation was carried out with a gradient of the following solvent mixtures, used as the mobile phase: Solvent A, Acetonitrile/5 mmol/L phosphate buffer, pH 5.6 (83:17); Solvent B, Acetonitrile/20 mmol/L phosphate buffer, pH 5.6 (50:50). The elution gradient program was the same as described earlier (17). The elution profile was monitored by flow-through detection of UV absorbance at 197 nm. The retention times of individual gangliosides were compared with the retention times of purified bovine brain–derived gangliosides (Matreya).

**Measurement of total lipid-bound sialic acid.** The concentration of isolated ganglioside expressed as lipid bound sialic acid was determined as described previously (18). Ganglioside (50 μL) was extracted with chloroform/methanol (2:1). Proteins are precipitated with phosphotungstic acid. Precipitated ganglioside was dissolved in boiling resorcinol-HCL reagent. The concentration of LSA is determined by the absorption at 580 nm.

**Mass spectrometric analysis of gangliosides.** Mass spectrometric analysis of the isolated gangliosides were done using a Waters Alliance 2690 HPLC connected to a Micromass Quattro-Ultima triple quadruple electrospray tandem mass spectrometer (LC-ESI-MS-MS). A Supelco Supelcosil LC-NH2 column (7.5 cm × 2.1 mm, 3 μm) was used as the stationary phase for the HPLC. Samples were dissolved in 83% acetonitrile and injected into the HPLC with a Waters-Alliance-2690 separation module autosampler. The column resolves the ganglioside mixtures into separate components each of which is then introduced into an electrospray needle at 0.3 mL/min. ESI-MS-MS spectra were taken using 0.1 mTorr argon as the collision gas at 60 eV.

**Analysis of T-cell apoptosis.** T-cell death was assessed by staining lymphocytes with trypan blue and counting the number of viable as well as the number of trypan blue positive cells.

Quantitation of DNA fragmentation in T cells was determined by the terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) assay. Fixed cells (1% paraformaldehyde) were stained and analyzed for apoptosis using the APO-BRDU kit system (Phoenix Flow Systems, San Diego, CA; ref. 19). Percentages of apoptotic T cells were obtained using quadrant analysis software (LYSSIS II, Becton Dickinson).

**Coculturing T cells with tumor cell lines or tumor-derived gangliosides.** T cells orJurkats were resuspended at a concentration of 2 × 10^6 cells/mL in complete RPMI 1640 before being added to the 100 mm dishes containing GBM cells at a 1.5:1 tumor/T-cell target ratio. Following a 72-hour incubation period, the nonadherent T cells were removed from the GBM monolayers and assessed for cell death by trypan blue and for apoptosis by TUNEL.

Blocking experiments were done with the addition of anti-CD70 antibody, anti-TNF-α, or Fas fusion protein at the time that T cells were incubated with the GBM monolayer. Activated T cells suspended at a concentration of 1 × 10^6 cells/mL in 1 mL of complete RPMI 1640 in 24-well plates were treated with 7 to 10 μg of isolated gangliosides (based on total sialic acid content). Seventy-two hours later, the T cells were assessed for cell death by trypan blue staining and the TUNEL assay.

**Inhibition of glucosylceramide synthase in glioblastoma multiforme cell lines by PPPP.** The glucosylceramide synthase inhibitor, 1-phenyl-2-hexadecanoylamino-3-pyryldino-1-propanol (PPPP), was added to the 50% confluent GBM cell lines to give the desired effective concentration of 1.0 μmol/L (20). Following a 5-day exposure of the GBM cells to PPPP, the drug was washed away, and the flask was replenished with fresh medium containing T cells. Gangliosides levels were evaluated by HPLC as previously described (21).

**Results**

Glioblastoma multiforme cell lines induce T-lymphocyte death that is most pronounced in activated cells. Glioma cells are likely responsible for the increase in lymphocyte death, because we show here that GBM cell lines can induce peripheral blood T-cell death (Fig. 1). Four long-term and three primary glioma lines were cocultured with resting T lymphocytes for 72 hours before assessing lymphocyte death. Four out of these seven GBM cell lines...
T cells. Columns, mean of four or more separate experiments; bars, ± SD.

Figure 1. GBM lines can induce T-cell death. A, seven GBM cell lines were cocultured with resting peripheral blood T cells from normal donors. After 72 hours, the nonadherent T cells were removed from the tumor monolayer and tested for cell viability. Four of the cell lines (CCF4, CCF52, U87, and D54) induced 17.6% to 33% death. Columns, mean of four or more separate experiments; bars, ± SD. B, seven GBM cell lines are cocultured with activated T cells for 72 hours before assessing lymphocyte viability. Five of the cell lines (CCF4, CCF52, U87, and D54) induced 38.6% to 54.5% death of activated T cells. Columns, mean of five or more separate experiments; bars, ± SD.

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Role of death receptors in glioblastoma multiforme–induced T-cell death. We examined the role that different TNF receptor (TNFR) family members play in GBM-induced T-cell death. This included TNFR family members that have a death domain in their cytoplasmic tails (Fas and TNFR1) and hence initiate apoptosis via the recruitment of adaptor proteins followed by activation of caspases (22).

TNF-α production by the GBM lines does not contribute to their apoptogenic activity, because none of the glioma expressed any detectable TNF-α mRNA or the secreted protein (data not shown). Furthermore, the addition of neutralizing antibody to TNF-α did not block the ability of GBM cells to induce apoptosis in T lymphocytes (data not shown); although this antibody did block T-cell death initiated by some renal cell carcinoma lines.4 These findings suggest that TNF-α does not play a significant role in the ability of the GBM lines used here to induce T-cell killing.

The role of FasL in GBM-induced apoptosis of T cells was also examined, because activated T cells express Fas/APO-1 (23) and some glioma lines express FasL (24). Staining with anti-FasL antibody, followed by flow cytometry analysis, a significant number of CCF52 (48%), U87 (27%), and D54 (95%) cells, which all induce T-cell death, expressed FasL (Fig. 2A). The nonapoptogenic GBM line, U138, and one of the apoptogenic GBM lines, CCF4, only stained weakly for FasL (10% and 5% positive cells, respectively; Fig. 2A). Addition of the inhibitory Fas fusion protein had no effect on the ability of CCF4 to induce T-cell death, because this line expressed very little FasL (Fig. 2A). However, the Fas fusion protein only had a modest ability to inhibit T-cell death initiated by the GBM lines (CCF52, 19% inhibition; U87, 19% and D54, 28%) in which a high percentage expressed FasL (Fig. 2A). We found similar results with Jurkats (data not shown). The effectiveness of FasFc to block (60%) T-cell death induced by anti-Fas antibody and by soluble FasL but not GBM-induced cell death suggests that GBM induced cell death is mostly FasL independent (Fig. 2A).

Role of CD70 ligand in glioblastoma multiforme–induced T-cell death. CD27 is a TNFR family member that can mediate apoptosis when activated by its cell surface ligand, CD70. Unlike TNFR1 and Fas receptor, CD27 lacks the cytoplasmic death domain (25). Instead, CD70 transduces its apoptotic signal through a cytoplasmic protein, Siva, which initiates apoptosis by mediating mitochondrial damage and caspase activation (26). Here we show that three of the apoptogenic GBM lines had a high percentage of CD70-positive cells, CCF52 (40%), U87 (47%), and D54 (46%; Fig. 2B). CD27 is a TNFR family member that can mediate apoptosis when activated by its cell surface ligand, CD70. Unlike TNFR1 and Fas receptor, CD27 lacks the cytoplasmic death domain (25). Instead, CD70 transduces its apoptotic signal through a cytoplasmic protein, Siva, which initiates apoptosis by mediating mitochondrial damage and caspase activation (26). Here we show that three of the apoptogenic GBM lines had a high percentage of CD70-positive cells, CCF52 (40%), U87 (47%), and D54 (46%; Fig. 2B). We found similar results with Jurkats (data not shown). The effectiveness of FasFc to block (60%) T-cell death induced by anti-Fas antibody and by soluble FasL but not GBM-induced cell death suggests that GBM induced cell death is mostly FasL independent (Fig. 2B).

We examined the role that different TNF receptors play in GBM-induced T-cell death. Anti-CD70 antibody, followed by flow cytometry analysis, a significant number of CCF52 (48%), U87 (27%), and D54 (95%) cells, which all induce T-cell death, expressed CD70 (Fig. 2B). The nonapoptogenic GBM line, U138, and one of the apoptogenic GBM lines, CCF4, only stained weakly for CD70 (10% and 5% positive cells, respectively; Fig. 2B). We found similar results with Jurkats (data not shown). The effectiveness of CD70 to block (60%) T-cell death induced by anti-CD70 antibody and by soluble FasL but not GBM-induced cell death suggests that GBM induced cell death is mostly FasL independent (Fig. 2B).

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Gangliosides expressed by glioblastoma multiforme lines contribute to tumor-induced T-cell death. Additional experiments determined whether gangliosides expressed by the GBM lines are involved in T-cell killing. To address this possibility, we tested whether reducing the expression of glycosphingolipids by GBM lines would impair their ability to induce T-cell apoptosis. The GBM lines were treated with PPPP, an inhibitor of glucosylceramide synthase, for 5 days before adding T cells for an additional 72 hours. PPPP pretreatment of U87 cells caused a 50% to 60% reduction in expression of GM3, GM2 GD3, and GD1a gangliosides as determined by HPLC analysis (Fig. 3A). A similar decrease in gangliosides expression by the other tumor lines cultured with PPPP was noted (data not shown). Pretreatment of GBM cell lines with PPPP did not alter the growth or the viability of the tumor cells. Five days after culturing 300,000 cells, the number of viable cells recovered from medium and PPPP-treated cells, respectively, was CCF4 $1.93 \times 10^6$ and $1.69 \times 10^6$, CCF52 $3.2 \times 10^6$ and $3.5 \times 10^6$, U87 $2.59 \times 10^6$ and $2.49 \times 10^6$, U138 $2.46 \times 10^6$ and $2.19 \times 10^6$, and D54 $2.75 \times 10^6$ and $3.058 \times 10^6$. However, tumor cell lines pretreated with PPPP (CCF4, CCF52, U87, and D54) were significantly less effective at inducing T-cell apoptosis as compared with untreated GBM lines (Fig. 3B). These show that PPPP has an inhibitory effect on glycosphingolipids synthesis in GBM cell lines, which consequently reduces their ability to kill T cells. The importance of GBM-derived gangliosides in T-cell killing is further highlighted by the demonstration that gangliosides isolated from the tumor lines and their supernatants were sufficient to induce cell death of activated T cells following 72 hours of treatment. Gangliosides isolated from the apoptogenic GBM lines and supernatants, but not the nonapoptogenic line, caused T-cell death ranging anywhere from 52% to 80% (with the apoptogenic lines' gangliosides; Fig. 4A) and 44% to 48% (with the supernatants' gangliosides; Fig. 4B). These gangliosides also induced DNA breaks as determined by the TUNEL assay, to an extent that varied depending on the cell type (CCF4, 29%; CCF52, 81%; U87, 35%; and D54, 31%; Fig. 4A). The concentration of gangliosides isolated from the GBM lines was determined by measuring total lipid bound sialic acid (18), and they ranged from 7 to 10 μg/mL. Based on the above findings, we propose that gangliosides are a second class of molecules involved in the GBM-induced T-cell killing.

(15%; Fig. 2D) as predicted, because only a low percentage (10%) of CCF4 cells express CD70.

Figure 2. CD70 but not FasL expression on GBM lines is important for inducing T-cell death. A, all four apoptogenic GBM lines and one nonapoptogenic line were assessed for surface expression of FasL by flow analysis following staining with anti-FasL antibody, CCF52, U87, and D54, which all induce T-cell death, stained positive for FasL, 48%, 27%, 95%, respectively, whereas CCF4 and the nonapoptogenic line U138 only showed low level of FasL staining. B, five GBM cell lines were cocultured with activated T cells for 72 hours in the presence of Fas fusion protein (blocking agent), fusion control, or medium alone. To show the efficiency of Fas-Fc, two positive controls, anti-Fas activating antibody, and supernatants from ecotropic retroviral packaging cells (GP + E 86) carrying human FasL-expressing retroviral particles (13) were used. Fas fusion protein only blocked cell death of activated T cells by 19% in CCF52 and U87 and 28% in D54. It had no effect with CCF4 or U138. C, GBM lines were stained with anti-CD70 antibody and the percentage of CD70-positive cells was assessed by flow cytometry. The three of the four cell lines that induce T-cell death had high percentage of CD70 (CCF52 40%, U87 47%, and D54 46%). Only 10% of the apoptogenic line, CCF4 expressed CD70. D, the same GBM cell lines were cocultured for 72 hours with activated T cells in the presence of CD70 blocking antibody, immunoglobulin G control, or medium alone. Thereafter, cell viability was determined by trypan blue staining. CD70 antibody showed partial protection against T-cell death with three of the apoptogenic lines (40% block with CCF52, 58% with U87, and 60% with U87). Only 15% protection was observed with CCF4 cells when incubated with anti-CD70 antibody. Similar results were observed in a second experiment.
HPLC analysis revealed that the gangliosides isolated from the different GBM lines consist of a mixture of gangliosides that vary in composition (Fig. 3A and Fig. 4C). Based on the elution times of standard bovine brain–derived gangliosides off the HPLC column, it was determined that the gangliosides isolated from the apoptogenic GBM lines (CCF4, CCF52, U87, and D54) were enriched in GM2 and GD1a compared with those isolated from the nonapoptogenic tumor lines (CCF3 and U138). Furthermore, CCF4 showed increased levels of GM1 (data not shown) when compared with other isolated GBM gangliosides. On the other hand, GD3 levels were relatively high in all of the GBM lines tested, including the nonapoptogenic lines. The apoptogenic lines, unlike the nonapoptogenic lines, also contained additional gangliosides that did not coelute with the purified standard brain-derived gangliosides (Fig. 3A and Fig. 4C). HPLC with online LC-ESI-MS-MS analyses employing parental scan confirmed that gangliosides expressed by CCF52 included GM2, ganglioside coeluting with GM2, GD1a, and GD3 (Table 1). We also analyzed the gangliosides shed by CCF52 with mass spectroscopy and found that they were mostly comprised of several molecular weights (MW) of GM2 and several gangliosides coeluting with GM2. However, the shed gangliosides had no detectable GD1a and GD3 which was observed in the gangliosides isolated from the CCF52 cells (Table 1).

To address which gangliosides expressed by the GBM are capable of inducing T-cell apoptosis, gangliosides isolated from CCF52 and U87 cell lines were subjected to HPLC separation, and individual fractions eluting off the column were tested for their induction of T-cell killing using activated T cells. Similar results were observed with gangliosides fractionated from both CCF52 and U87 showing two major peaks of apoptotic activity. The initial peak coeluted off the HPLC column with a retention time similar to the bovine brain standard GM2, whereas the other peak coeluted with a retention time near GD1a and GD3 (Fig. 5A). Mass spectroscopy employing parental scan showed that the masses for GM2 (Fig. 5B) and GD1a/GD3 (data not shown) are present in the first and second peaks, respectively. ESI-MS-MS analysis was used to determine if there is more than one ganglioside in the HPLC fraction that contained GM2 by noting if there were several parent ion peaks that yield the characteristic daughter ion mass of N-acetylneuraminic acid (NeuAc, 291.09 MW). Indeed, we did detect the masses of GM2 (GM2 masses 1,382 and 1,410). The parental scan also detected ganglioside masses distinct from GM2 but that elute with GM2: 1,466, 1,465, 1,371, 1,354, and 1,352 (Fig. 5B).

Based on the result that fractions with the most T-cell killing contained multiple gangliosides including GM2, GD1a, and GD3, we wanted to determine if combination of gangliosides would improve the induction of T-cell apoptosis. Therefore, we tested purified bovine gangliosides individually and in combination to assess T-cell killing effect. Activated T cells were treated with purified bovine gangliosides GM1, GM2, GM3, GD1a, and GD3. All

**Figure 3.** A reduction in glycosphingolipid synthesis by the GBM lines partially blocked their ability to induce T-cell death. A, the U87 cell line was incubated with the glucosylceramide synthase inhibitor PPPP (1 μmol/L) for 5 days. Thereafter, HPLC analysis was performed on gangliosides extracted from the same number of U87 cells (4 × 10⁶) pretreated or not with PPPP. The retention time of gangliosides isolated from U87 was compared with that of purified bovine brain derived ganglioside run in the same experiment. PPPP treatment caused a 2-fold reduction in ganglioside expression by U87, including GM3, GM2, GD3, and GD1a when compared with untreated U87. B, activated T-cell were cocultured with GBM cell lines treated or not with PPPP (for 5 days) and assessed for tumor-induced cell death by trypan blue staining. T-cell death was reduced by 40% when lymphocytes were cocultured with CCF4, CCF52, and D54 cells pretreated with PPPP and 50% when cocultured with PPPP treated U87. Columns, mean of three different experiments; bars, ±SD.
Figure 4. Gangliosides isolated from GBM lines induced T-cell death, with the apoptogenic GBM lines expressing elevated levels of select gangliosides compared with the nonapoptogenic GBM lines. Activated T cells were incubated with (A) isolated gangliosides from five GBM cell lines or with (B) isolated gangliosides from GBM supernatant. After 72 hours, T cells were harvested and assessed for apoptosis by staining with trypan blue and by TUNEL. Gangliosides isolated from all the apoptogenic GBM lines or their supernatant but not the nonapoptogenic line caused T-cell death anywhere from 44% to 80% by trypan blue staining and 29% to 81% by TUNEL. The final concentration of isolated ganglioside’s lipid-bound sialic acid in this experiment ranged from 7 to 10 μg/mL. C, gangliosides isolated from an apoptogenic line (CCF52) and a nonapoptogenic tumor line (CCF3) were then subjected to HPLC fractionation. The retention times of gangliosides derived from the GBM were compared with the retention times of purified bovine brain gangliosides. HPLC analysis revealed that the gangliosides isolated from the apoptogenic GBM line CCF52 showed increased levels of GM2 and GD1a when compared with those isolated from the nonapoptogenic tumor line CCF3. Similar increases in GM2 and GD1a expression were observed with gangliosides isolated from other apoptogenic GBM lines, (CCF4, U87, and D54) but not from the nonapoptogenic line U138. It was noted that GD3 was expressed to similar levels by all of the GBM lines.
but GM3 were capable to induce T-cell killing at high concentration (150 μg/mL; Fig. 5C) but none at low concentration (40 μg/mL; Fig. 5D). However, when GM1, GM2, GD1a, and GD3 were used in combination at low concentration (10 μg/mL each for a total of 40 μg/mL) a synergetic effect in T-cell killing was observed (Fig. 5D).

For some GBM lines (CCF52, U87, and D54), the ability to induce T-cell death involves both CD70 and gangliosides expressed by the tumors. Whether blocking CD70 function and ganglioside expression together would provide better protection from GBM-induced apoptosis than blocking either one alone was examined. The GBM lines were pretreated with PPPP for 5 days before adding anti-CD70 antibody and activated T cells to the tumor monolayer. As previously shown, pretreatment of GBM lines with either PPPP or the addition of CD70 antibody partially blocked T-cell death. For the three lines that expressed CD70, blocking both CD70 function and ganglioside synthesis provided greater protection than blocking either one alone. This combined effect seems additive. As expected, there was little protective value observed by combining PPPP and anti-CD70 antibody in coculture experiments with the CD70-negative GBM line (CCF4; Fig. 6).

**Discussion**

One role of T cells is to recognize and respond to neoplastic cells anywhere in the body including the central nervous system (27–29). Immune effector cells do enter the brain in response to molecules expressed by the endothelial cells and astrocytomas (30). Even when the immune system is stimulated in the periphery, immune effector mechanisms are activated in the brain as well (31). Furthermore, between 30% to 60% of gliomas are infiltrated with inflammatory cells (28, 32) with a predominance of CD8+ T cells (33). Whereas lymphocyte infiltration does occur in gliomas (27–29), a significant number of these immune cells undergo apoptosis (5, 34). Thus, tumor progression occurs despite lymphocyte infiltration, likely due to tumor-induced immune dysfunction (27).

Patients with gliomas are characterized by systemic immune dysfunction, as shown by impaired cell-mediated immunity, lymphopenia (2, 3, 6), and inability to mount delayed-type hypersensitivity reaction (3). The degree of immunosuppression observed in glioma patients may correlate with tumor size and grade (35, 36). Interestingly, peripheral T cells are observed to regain some functions following removal of the primary tumor, although they lose it again upon recurrence (37). These findings suggest that soluble products secreted by gliomas contribute to the lymphocyte dysfunction observed within the tumor and peripheral blood.

The immunosuppression initiated by the high-grade gliomas may be a barrier to the development of an effective immune response to GBM immunotherapy. Our findings reveal that many but not all GBM lines can induce apoptosis in peripheral blood T cells from healthy individuals and that activated T lymphocytes are significantly more susceptible to tumor-induced apoptosis than resting cells. Most relevant is the observation that the GBM lines we tested kill T cells by two different mechanisms. One involves a receptor-mediated pathway of apoptosis that is initiated by the ligand CD70 expressed on some of the GBM lines. The other pathway is receptor independent and is mediated by select gangliosides expressed on the GBM lines. Interestingly, three of the GBM lines induced T-cell death by both mechanisms, whereas another line induced death through a receptor-independent ganglioside-mediated pathway.

Several different molecules have been proposed to mediate immune escape by human tumors including GBMs. Fasl has been reported to be expressed by various histologically distinct tumors and thereby can induce apoptosis of FasR-positive T cells (38–40). However, this mechanism remains controversial due to conflicting data regarding FasL expression on some tumor types (41). Furthermore, it has been suggested that T-cell apoptosis may not be initiated by FasL-positive tumors in coculture experiments but may result from FasL-expressing T cells interacting with FasR-expressing T cells (41). Other groups have described FasL expression on high-grade glioma specimens (5, 24), GBM cell lines (42), and GBM tissue endothelial cells (43). Indeed, we observed varying levels of FasL on three of the apoptogenic GBM lines (CCF52, U87, and D54). However, Fas fusion protein only blocked a modest amount of T-cell death, suggesting that for these GBM cell lines, FasL expression is not a major mechanism of tumor escape. Likewise, TNF-α does not seem involved in the GBM-induced apoptosis of T cells, although under certain conditions, it can mediate apoptosis via the TNFRF (44). In fact, none of our GBM cell lines expressed either TNF-α mRNA or protein, which is not surprising because gliomas have been found to secrete TNF-α only upon stimulation (45). Furthermore, the addition of anti-TNF-α neutralizing antibody did not block T-cell death induced by GBM lines.

One mechanism by which some GBM cell lines can kill T cells is through their expression of CD70, a surface protein that is the ligand for CD27, a member of the TNFR family (46). CD70 is normally expressed on activated B and T lymphocytes, whereas the CD27 receptor is constitutively expressed on both resting and activated lymphocytes (47). The interaction of CD70 with CD27 can provide a cosstimulatory signal important for T-cell activation (48). However, CD70/CD27 interaction can also lead to T-cell apoptosis, probably due to persistent stimulation (49, 50). We now show that CD70 expressed on some GBM lines can also induce death of T lymphocytes as shown by the observation that anti-CD70 antibody when added to cocultures partially blocked T-cell killing. Our results are similar to a recent report (50) which showed immune escape of high-grade gliomas via the interactions of CD70 ligand expressed on glioma cells and CD27 expressed on T cells to induce lymphocyte apoptosis. The one difference between our findings and those of Wischhusen et al. (50) was with the U138 cell line that was CD70 positive and apoptogenic in their
Figure 5. HPLC fractionation of CCF52 and U87 gangliosides revealed two distinct peaks of apoptogenic activity. A. Gangliosides isolated from CCF52 and U87 cell lines were subjected to HPLC separation and individual fractions were tested for their induction of apoptosis using activated T cells (following a 72-hour incubation period). Both CCF52 and U87 fractionation showed two major peaks of T-cell killing activity. The initial peak had a retention time similar to the bovine brain standard GM2, while the other peak had a retention time near GD1a and GD3. All fractions and the crude gangliosides have the same amount of ganglioside (7 μg/mL). B. GM2 and other gangliosides coeluting with GM2 are present in the first apoptogenic peak following HPLC fractionation of the GBM ganglioside CCF52. HPLC with online LC-ESI-MS-MS analyses, employing parent scan, was used to define the gangliosides masses present in the first peak that had apoptogenic activity against T cells (fraction 6). The parent scan (inset) showed that this HPLC fraction 6 contains several parent ion peaks including two that are characteristic of GM2 (GM2 masses 1,382 and 1,410). Additionally, this fraction contained five predominant masses that correspond to gangliosides distinct from GM2 but that coelute with GM2 and have the masses of 1,466, 1,465, 1,371, 1,354, and 1,352. C. GM1, GM2, GD1a, and GD3 purified bovine gangliosides but not GM3 induce 60% to 70% T-cell killing at 150 μg/mL. D. At lower concentration (40 μg/mL), individual gangliosides are incapable of killing T cells; whereas in combination (10 μg/mL each to make a total of 40 μg/mL), they can induce 30% T-cell death.
GM2 was present but it also showed that several other unidentified
spectrometry analysis of the first peak did reveal that the mass for
other peak had a retention time similar to GD1a and GD3. Mass
significant T-cell death activity. One peak had a retention time
GBM lines (CCF52 and U87) identified two distinct peaks with
included those with retention times of GM2 and GD1a.
This reduction in ganglioside expression by the nonapoptogenic lines
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HPLC analysis showed reduced expression of several ganglioside
their supernatants did not induce significant T-cell killing, and their
gangliosides isolated from the two nonapoptogenic tumor lines and
apoptosis in T cells at relatively low concentrations. As expected,
cooperate to induce T-cell death. Activated T cells were cocultured with GBM cell
lines with or without anti-CD70 blocking antibody (10 μg/mL), glucosylceramide
synthase inhibitor PPPP (1 μmol/L), or a combination of anti-CD70 and PPPP.
GBM lines were pretreated with PPPP for 5 days before adding anti-CD70 blocking
antibody and activated T cells to the GBM monolayer. Pretreatment of GBM lines
with either PPPP or the addition of CD70 antibody partially blocked T-cell death.
In the three lines that expressed high CD70 (CCF52, U87, and D54), combined
treatment of anti-CD70 and PPPP provided greater protection than blocking either
one alone.

The data presented here indicate that gangliosides expressed by
the apoptogenic GBM lines can also mediate T-cell death independently of CD70. We initially showed that the glucosylceramide synthase inhibitor, PPPP, blocked ganglioside expression on
GBM lines by 50% and comparably reduced their ability to induce T-
cell death (50%), suggesting that gangliosides play a significant role
in T-cell killing. Because PPPP additionally blocks the synthesis of
other glycosphingolipids, it was not possible to conclude from these
experiments that gangliosides were the major lipid involved in T-cell
death. Direct involvement of gangliosides in T-cell killing was
supported by the observation that gangliosides isolated from
apoptogenic tumor lines and their supernatants were able to induce
apoptosis in T cells at relatively low concentrations. As expected,
gangliosides isolated from the two nonapoptogenic tumor lines and
their supernatants did not induce significant T-cell killing, and their
HPLC analysis showed reduced expression of several gangliosides
when compared with the apoptogenic GBM lines (Fig. 3A and Fig. 4C).
This reduction in ganglioside expression by the nonapoptogenic lines
included those with retention times of GM2 and GD1a.

Analysis of individual HPLC fractions from two apoptogenic
GBM lines (CCF52 and U87) identified two distinct peaks with
significant T-cell death activity. One peak had a retention time
similar to bovine brain–derived ganglioside GM2, whereas the
other peak had a retention time similar to GD1a and GD3. Mass
spectrometry analysis of the first peak did reveal that the mass for
GM2 was present but it also showed that several other unidentified
gangliosides coeluted with GM2. Analysis of isolated gangliosides of
CCF52 supernatant by mass spectrometry also showed high
concentration of GM2 and several other gangliosides coeluting
with GM2. Using commercial bovine gangliosides, we determined
that GM1, GM2, GD1a, and GD3 induced T-cell death at high concentration (150 μg/mL) but not at low concentration
(40 μg/mL). However, when combined, they achieved a synergistic
effect and an appreciable killing of T cells at 40 μg/mL. This finding
supports our HPLC and mass spectroscopy results, suggesting that
multiple gangliosides contribute to T-cell death induced by GBM
lines. It was also shown that, in the HPLC fractionation data, the
two peaks with apoptogenic activity were not as potent as the
unfractionated gangliosides isolated from CCF52 suggesting
maximal T cell killing is the result of multiple gangliosides. It may
be that the inability of gangliosides isolated from CCF3 (10 μg/mL)
to induce apoptosis is due to the fact that CCF3 expressed less
GM2 (plus GM2 like) and GD1a than the apoptogenic CCF52 line
although both had similar amounts of GD3. The fact that the
isolated GBM gangliosides (containing multiple gangliosides) were
able to kill at much lower concentrations (7-10 μg/mL) than the
individual purified bovine brain–derived gangliosides further
suggest that there is cooperation among individual gangliosides
to induce potent apoptogenic activity.

Gangliosides are overproduced in a number of different tumor
types including gliomas (51–53). Gangliosides are known to be shed
from tumors and can be detected in the peripheral blood of cancer
patients. Elevated GD2 levels in neuroblastoma patients seemed to
correlate with tumor progression and a lower survival rate (54).
Although gangliosides play important roles in cell differentiation,
cell adhesion (55), and modulation of receptor function (56) and
participate in tumor progression (57), they also inhibit host
immunity (12, 21). Administration of tumor-derived GM1b
inhibited the in vivo development of antitumor immune responses
and promoted malignant growth (12). Gangliosides isolated from
different tumors are known to inhibit immune responses (21, 58),
which may be related to their capacity to suppress T-cell
proliferation and Th-1 cytokine production in vitro (15, 59).
Gangliosides have also been shown to induce apoptosis in various
cell types including lymphocytes (15).
GBM tumors are known to either overexpress or synthesize modified forms of GD3, GD2, GM1, GM2, 3′iso-LM1, and 3′6′iso-LD1 (8, 10, 53, 60). Others have reported that gangliosides (mostly GD3 and GM3) are shed into the serum of GBM patients (8, 61). This shedding of gangliosides by GBMs may partly explain lymphopenia, apoptosis, and depressed T-cell function in the periphery of these patients (3, 62, 63). Although the responsible agents were not defined in these studies, it was proposed that perhaps unknown or known molecules such as transforming growth factor-β1, 2, prostaglandin E2, IL-10, or even other unique molecules were involved (64–66). Our findings suggest that tumor-associated CD70 and gangliosides expressed and shed by GBM, both mediate T-cell apoptosis, providing the glioblastomas mechanisms by which they both evade host immunity and hence can continue their progressive growth.

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References


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