Gangliosides Expressed by the Renal Cell Carcinoma Cell Line SK-RC-45 Are Involved in Tumor-induced Apoptosis of T Cells

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ABSTRACT

It is now understood that the genetic plasticity of cancer cells can lead to alterations that confer selective growth advantages to the tumor, some of which play a role in immune escape. A number of mutations resulting in tumor cells from host immune defenses have been well characterized but more recent studies suggest that a variety of tumors can also express products that are actually toxic for the immune effectors. A component of this tumor-induced T-cell death has been attributed to receptor-mediated apoptosis. Some tumors, however, synthesize soluble factors that mediate similar effects. In this regard, we previously showed that supernatants from explanted renal cell carcinoma (RCC) tumors sensitized normal T cells to activation induced cell death, and the responsible products had the features of gangliosides. We have also shown that renal tumor lines, including SK-RC-45, induce apoptosis of both Jurkat cells and normal T lymphocytes. Here, we used the ganglioside synthesis inhibitor PPPP to define the role of gangliosides in RCC cell line (SK-RC-45)-mediated T cell and Jurkat cell apoptosis and to elucidate the proapoptotic molecular events by which the glycosphingolipids produce their effects. The ganglioside-synthesizing SK-RC-45 line stimulated the TUNEL (terminal deoxynucleotidyl transferase-mediated nick end labeling) positivity of cocultured T cells by a mechanism that involved decreasing lymphocyte activation expression levels of Bcl-2 and Bcl-XL, inducing cytochrome c release from their mitochondria and activating caspases 9 and 3. These proapoptotic events were partially or completely abrogated when tumor cells were pretreated with PPPP for 5 days before the SK-RC-45/T lymphocyte coinubation, a regimen that reduced tumor-associated ganglioside levels by 70–80%. Our results suggest that gangliosides may be key mediators of RCC-induced T-cell apoptosis and imply that they contribute to the T-cell dysfunction in the tumor microenvironment.

INTRODUCTION

There are multiple lines of evidence to suggest that cancer patients do initiate immune reactions to their tumors (1–3). It is apparent that these responses are generally ineffective, however, because most tumors continue to grow progressively (4, 5). A number of the mechanisms by which tumors evade immune derive from the genetic instability of the malignant cells (6). Decreased HLA expression and genetic aberrancies in antigen processing, antigen presentation (7–9), or caspase activation (3, 10) are among the better-described passive strategies by which specific tumor cells have been shown to resist an immune response. Less characterized or understood, however, are the tumor cell alterations that encode more aggressive modes of resistance, several involving actual destruction of the immune effectors (5, 11–13). Indeed, in situ TUNEL analysis performed on tissue sections of histologically diverse tumors, including renal cell carcinomas, indicates that 10–20% of the infiltrating T cells are apoptotic (11, 12). Several laboratories have now confirmed that it is the tumor cells themselves that mediate these effects because when cocultured in vitro, cancer cell lines clearly can be shown to induce T lymphocytes to undergo all of the physiological changes associated with apoptosis and activation-induced cell death (13–15).

Studies on the mechanisms by which tumor cells induce T-cell apoptosis have focused primarily on the receptor-dependent pathway. Indeed, a number of reports suggest that histologically disparate tumors aberrantly express elevated levels of various tumor necrosis factor-related ligands such as FasL, TRAIL, and CD70, which kill T cells in a receptor-dependent fashion (12, 16–18). Soluble, tumor-derived factors may also have a role in inhibiting the immune response, however. We reported that conditioned media taken from in vitro cultured, explanted RCC tumors could inhibit nuclear factor κB activation of cocultured T cells (19), and identified an active component of the supernatant as a ganglioside. More recently, we showed that gangliosides isolated from some renal tumors can sensitize T cells to activation-induced cell death (20). Here, we examined whether RCC gangliosides might contribute to the ability of tumor cells to induce T-cell apoptosis.

Gangliosides are structurally diverse acidic glycosphingolipids that are present in the outer leaflet of plasma membranes (21). Although ubiquitously expressed, gangliosides do show cell type specificity and differentiation-related expression patterns (22). Although once considered to have a strictly structural function, there is increasing evidence that gangliosides serve as signaling intermediates in the regulation of multiple cellular functions in some instances even associating with and modulating the activities of various receptors (23–26).

Many tumors exhibit enhanced synthesis of select gangliosides, some of which are shed into the tumor microenvironment (27, 28). As early as 1977, Kloppe et al. (29) demonstrated that gangliosides and ganglioside biosynthetic enzymes are elevated in tumors and that cancer patients and tumor-bearing animals have elevated levels of glycolipid-bound sialic acid in their sera. Malignant melanomas and neuroblastomas, for example, overexpress GD3, GD2, and GM2, whereas renal cell carcinomas display increased levels of GD1a, GM1, and GM2 as compared with cells of the normal kidney (22). Some stem-derived gangliosides are immunosuppressive, and Dr. Stephen Ladisch has extended Black’s work (27) by publishing on the ability of those molecules to inhibit specific components of the immune response (30–33), thereby enhancing tumor formation and progression (34–36). In this context, RCC has been shown to synthesize several unique disialogangliosides, which seemingly promote the metastatic capabilities of that tumor (37).

In the studies reported here, we used an inhibitor of ganglioside synthesis to demonstrate the significant role those glycosphingolipids play in mediating RCC tumor-induced T-cell apoptosis. In coculture experiments, the renal cancer cell line SK-RC-45 induced the apoptotic response of T cells (38). Tobacco smoke, which contains a number of compounds that can promote the growth and metastatic capabilities of tumors (39), has been shown to enhance the expression of both gangliosides and disialogangliosides (38, 40). Furthermore, we observed that treatment of SK-RC-45 cells with a ganglioside biosynthesis inhibitor, PPPP (41), reduced the levels of GD1a, GD2, and GM2 in those cells (42). Thus, we concluded that gangliosides play a role in RCC tumorigenesis and metastasis.

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3 The abbreviations used are: TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; RCC, renal cell carcinoma; PPPP, 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol; IL, interleukin; HPTLC, high-performance thin-layer chromatography; TIL, tumor-infiltrating lymphocyte; ROS, reactive oxygen species; AICD, activation induced cell death.

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ptotic death of T cells by a mechanism that included decreasing the lymphocytic expression levels of Bcl-2 and Bcl-xL, with coincident mitochondrial release of cytochrome c, and caspase activation. The ability of the ganglioside synthesis inhibitor PPPP to attenuate the effects of SK-RC-45 on T-cell TUNEL positivity, Bcl-2/Bcl-xL expression, cytochrome c release, and caspase activation suggests that those glycosphinolipids play an integral role in tumor-induced T-cell apoptosis and immunosuppression.

**MATERIALS AND METHODS**

**Reagents.** Anti-cytochrome c was a mouse polyclonal antibody (BD PharMingen, San Diego, CA) used at a 1:5000 dilution to detect tumor-induced cytochrome c release from T-cell mitochondria. Anti-Bcl-2 was a murine monoclonal IgG1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) used at 2 μg/ml, and anti-Bcl-xL was a polyclonal rabbit IgG antibody (Santa Cruz Biotechnology) used at the same concentration. Anti-DFP-45 was a goat polyclonal IgG (Santa Cruz Biotechnology), and anti-human procaspase 9 was a murine monoclonal IgG (Oncogene Research Products, Boston, MA), both used at 2 μg/ml. A murine antiactin monoclonal antibody (Santa Cruz Biotechnology) also used at 2 μg/ml was the control for these experiments. Secondary horseradish peroxidase-conjugated sheep antimouse and donkey antirabbit antibodies were purchased from Amersham (Arlington Heights, IL). Monoclonal anti-CD3 antibody (OKT3, Ortho Biotech, Raritan, NJ) and monoclonal anti-CD28 antibody (Becton Dickinson Immunocytometry Systems, San Jose, CA) were used for the stimulation of T lymphocytes. Human recombinant IL-2 (Aldeeslukin [Proleukin], Chiron Corporation, Emeryville, CA) was used at 200 units/ml to maintain the viability of activated T cells. The ganglioside synthesis inhibitor PPPP was purchased from Matreyra (Pleasant Gap, PA).

**Isolation of T Cells from Peripheral Blood and Tumor.** Peripheral blood was obtained from healthy volunteers with informed consent. Blood was centrifuged over a Ficoll-Hypaque density gradient (Amersham Pharmacia Biotech AB, Upsalla, Sweden) to obtain total leukocytes from which T cells were purified by negative magnetic selection using microbeads coated with antibodies to CD14 (macrophage), CD16 (natural killer cells), CD19 (B cells), CD56 (natural killer cells), and glycophorin A (RBCs; Stem Cell Technologies, Vancouver, British Columbia, Canada). The T-cell isolation procedure yielded cells that were >95% positive for CD3 as defined by immunocytochemistry.

Stimulation of T cells with cross-linked anti-CD3 antibody (OKT3) plus anti-CD28 antibody was performed by first coating flasks with 10 μg/ml of anti-CD3 and 5 μg/ml of anti-CD28 antibody in 1 ml Tris buffer (pH 8.0) for 1 h. The flasks were washed twice with RPMI to remove unbound antibodies, and cells were added to a density of 1 × 10^6/ml for stimulation. Cells were activated for 3 days, at which point lymphocytes were transferred to fresh flasks in which they were expanded for 2–3 weeks in the presence of 200 units/ml IL-2 before use.

**Cell Lines.** The Jurkat leukemic T-cell line was purchased from American Type Culture Collection (Manassas, VA) and was maintained in complete medium (RPMI 1640; BioWhittaker, Walkersville, MD) supplemented with 10% FCS (HyClone, Logan, UT), 2 mm t-glutamate, 50 μg/liter gentamicin, 100 mM MEM sodium pyruvate solution, and 10 mM MEM nonessential amino acid solution (Life Technologies, Inc., Grand Island, NY). The well-characterized, long-term RCC line (SK-RC-45; Ref. 38) was obtained from Dr. Neil Bander (The New York Hospital, Cornell University Medical College). These cells were maintained in complete RPMI medium at 37°C with 5% CO₂ and were allowed to reach confluence in 150-mm dishes before use in coculture experiments with activated, peripheral blood T cells or Jurkat cell populations. The B-cell myeloma lines RPMI 8226 and U266 were a generous gift of Dr. Maryam Zamanian (Cleveland Clinic Foundation).

**Inhibition of Glucosylceramide Synthase in SK-RC-45 Cells by PPPP.** The ganglioside synthesis inhibitor PPPP was dissolved in ethanol to a concentration of 1 mM and kept as a stock solution at −20°C. To inhibit glucosylceramide synthesis, the stock solution was warmed to 37°C, diluted first 1:10 in culture medium, and then again 1:100 into the 50% confluent SK-RC-45 cell cultures to give the desired effective concentration of 1.0 μM PPPP (39). The final concentration of ethanol in the culture media was −0.1% (v/v). Preliminary studies revealed that a 5-day treatment of SK-RC-45 with 1 μM PPPP most effectively inhibited ganglioside synthesis by the tumor cell line, and the results are consistent with the findings of others (39). After a 5-day exposure of the SK-RC-45 cells to PPPP, the drug was washed away, and the flask was replenished with fresh media containing Jurkat cells or activated T cells. After coculture, the nonadherent T-cell targets were harvested and processed for TUNEL analysis to assess tumor-induced T-cell apoptosis and for Western analysis to analyze tumor-induced alterations in proapoptotic protein expression by T cells.

**Ganglioside Isolation and HPTLC Analysis.** Gangliosides were isolated from PPPP-pretreated or untreated SK-RC-45 tumor cells essentially as described before (35). Briefly, cells were trypsinized, pelleted, lyophilized, and then extracted twice with chloroform-methanol (1:1) for 18 h at 4°C with stirring. The combined extracts were reduced to 25% of their original volume and cooled to −20°C overnight before removing insoluble material by centrifugation at 1000 × g for 10 min. The lipid-supernatant supernatant was dried under nitrogen, lyophilized, and then partitioned three times in 10 ml of disopropyl ether/1-butanol/0.1% aqueous NaCl (40). The lyophilized, final aqueous phase was dissolved in 10 ml of ddH₂O after which, salts and small molecular weight impurities were removed using a Sephadex G-25 column. Isolated gangliosides eluting in the void volume were lyophilized and redissolved in chloroform:methanol for HPTLC. The HPTLC analysis of gangliosides was performed using 10 × 0.5 cm precoated LHPKD silica gel – 60 Å HPTLC plates (Whatman, Inc., Clifton, NJ). The plates were developed in isopropanol-50 mM KCl (aqueous)-methanol (59:20:9, v/v/v). Gangliosides were visualized as purple bands with the resorcinol-HCl reagent. Bovine brain gangliosides (GT1b, GD1b, GD1a, GM1, GM2, and GM3; Sigma) were run on lanes of the HPTLC plates as standards.

**Induction of Peripheral Blood T-Cell and Jurkat Cell Apoptosis by SK-RC-45 Cells.** The ability of PPPP-treated or -untreated tumor cells to induce apoptosis of activated, peripheral blood T cells or Jurkat cells was assessed by incubating target cells with 150 mM tissue culture RPMI containing 30 × 10⁶ SK-RC-45 cells with the target cells for 48 h (Jurkat cells) or 72 h (activated T cells) at a 1:3:1 tumor:target cell ratio. After coculture, the nonadherent target cells were removed from the SK-RC-45 monolayers by gentle washing and then were processed for either TUNEL analysis or trypan blue exclusion (41).

**Analysis of DNA Fragmentation by TUNEL Analysis.** Cells were fixed in 1% paraformaldehyde and were stained and analyzed for apoptosis using the APO-BrdUrd system (Phoenix Flow Systems, San Diego, CA). Briefly, cells were labeled with 50 μl of DNA solution containing 10 μM of terminal deoxynucleotidyltransferase reaction buffer. Cells were rinsed and resuspended in 0.1 ml of a solution containing fluorescein PRB-1 antibody. Pro-pidium iodide/RNase A solution (0.5 ml) was added to each sample before incubation at room temperature for 30 min. Flow cytometric analysis was performed with a FACStar Plus (Becton Dickinson, Franklin Lakes, NJ) set to measure 10,000 events. T cells incubated in media served as negative controls, whereas HL60 promyelocytic leukemia cells induced to apoptosis with camptothecin were positive controls for the experiments. The percentages of apoptotic T cells were obtained using quadrant analysis software (Lysis II; Becton Dickinson).

**Cell Lysates and Analysis of Protein by Western Blotting.** Cell pellets were resuspended in lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT] containing protease inhibitors (5 μg/ml antiprotein, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonflouride, 100 μg/ml pefabloc, and 100 μg/ml chymostatin) in a final volume of 150 μl for 15 min at 4°C. After adding 10 μl of a 10% NP40 solution [20 mM Tris base (pH 7.4; Sigma) to the pellets, Eppendorf tubes were vortexed vigorously, centrifuged at maximum speed, and the cytoplasmic extracts collected, aliquoted, and stored at −80°C until use. Protein amounts were determined using an equal volume of 2× Laemml buffer, boiled, and resolved on 12% SDS-PAGE gels. After transfer to nitrocellulose membranes by electroblotting (Bio-Rad, Richmond, CA) as previously described (42), the blots were blocked by overnight incubation with 5% nonfat dry milk in Tris/boric acid/sodium chloride/Tween 20 and subsequently probed with the specific primary antibodies described above. The immunoreactive proteins were visualized using horseradish peroxidase-linked secondary antibodies and enhanced chemiluminescence (ECL Western blotting kit; Amersham).

**Cytochrome c Assay.** For analysis of cytochrome c release from target cell mitochondria after coculture of the lymphocytes with tumor cell monolayers,
1 × 10^7 lymphocytes were spun down, washed once with ice-cold PBS, and once with ice-cold mitochondria isolation buffer [20 mM HEPES-KOH, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, and 250 mM sucrose, containing protease inhibitors (5 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μM phenylmethylsulfonyl fluoride, 100 μg/ml pefabloc, and 100 μg/ml chymostatin)]. The cell pellet was resuspended in 100 μl of mitochondrial isolation buffer and, after 20 min on ice, was homogenized with a Dounce homogenizer. The homogenate was centrifuged at 750 × g for 20 min, and the supernatant, which contained released cytochrome c, was assessed for protein concentration. Equivalent amounts of protein were then mixed with an equal volume of 2× Laemmli buffer, boiled, resolved on 12% SDS-PAGE gels, and processed for Western analysis using a murine polyclonal anticytochrome c antibody as described above.

RESULTS

RCC Cell Line SK-RC-45 Induces Apoptosis of T Cells. When activated, peripheral blood T cells from normal donors were cocultured with the RCC tumor cell line SK-RC-45, a considerable percentage of the lymphocytes were induced to undergo apoptotic cell death by 72 h of incubation. An average of 19% (n = 3) of the activated T lymphocytes exposed to the RCC cell line underwent DNA breakage and became TUNEL positive (Fig. 1A), which is consistent with the change in T-cell viability, assessed using trypan blue exclusion (average, 24%; Fig. 1B).

Additional studies examined the signaling events associated with SK-RC-45-induced T-cell apoptosis. Although a number of reports have suggested that multiple tumor types can induce T-cell apoptosis in a receptor-dependent fashion (11, 12, 17, 18, 43), evidence is accumulating that other tumor-derived products can also induce apoptosis through a receptor independent, mitochondrial pathway (44, 45). Given the type II nature of Jurkat cells and T lymphocytes (46–48), mitochondrial release of cytochrome c is requisite to apoptosis induced through both pathways: it provides the needed amplification of receptor-mediated signals and actually initiates the caspase cascade when apoptotic signals are received through the mitochondria directly (45, 49, 50). Indeed, when lysates of activated T cells exposed or not to SK-RC-45 for 72 h were assessed by Western analysis for cytochrome c, it was clear that the tumor cell line stimulated the cytosolic accumulation of cytochrome c in the target lymphocytes, as well as activation of both caspase 9 (measured as loss of anti-caspase 9 reactivity) and caspase 3 (as assessed by cleavage of its substrate, DFF-45), in the same time frame (Fig. 2). Also evident after 72 h of coinoculation with SK-RC-45 was a decrease in lymphocyte expression levels of the Bcl-2 and Bcl-XL proteins (Fig. 2), significant, perhaps, given the important roles of these antiapoptotic molecules in maintaining the integrity of mitochondrial membranes and an intact electron transport chain (51). Coculture with the renal cancer cell line induced comparable changes in Jurkat T lymphocytes. When assessed by TUNEL analysis after 48 h of coinoculation with SK-RC-45, it was evident that the tumor cells had caused 56% (±SD, n = 3) of the Jurkat cells to undergo apoptosis (Fig. 3A). Additionally, as was the case with normal, activated T lymphocytes, this RCC-stimulated apoptosis was associated with both the cytosolic accumulation of cytochrome c and decreased cellular expression levels of Bcl-2 and Bcl-XL by the affected Jurkat cells (Fig. 3B). It should be noted that SK-RC-45-induced apoptosis of both Jurkat cells and activated T lymphocytes was prevented when the coinoculation took place in the presence of pancaspase inhibitor III (data not shown). In contrast to these results with Jurkat cells and normal, peripheral blood T lymphocytes, the myeloma lines U266 and RPMI 8226 demonstrated essentially complete resistance to a 72-h coinoculation with SK-RC-45.

Inhibition of Ganglioside Synthesis by SK-RC-45 Partially Abrogates Tumor-mediated T-Cell Apoptosis. Previous work from our laboratory demonstrated that gangliosides isolated from explanted RCC tumors inhibit nuclear factor xB activation and can sensitize normal peripheral blood T cells to activation induced cell death (19, 20). To assess the involvement of gangliosides in SK-RC-45-mediated apoptotic death of cocultured T cells and Jurkat cells, we asked whether pretreatment of tumor cells with the ganglioside synthesis inhibitor PPPP would affect the capacity of the tumor line to induce apoptosis of coincubated lymphocytes. First, however, to ascertain the
actual effectiveness of PPPP in down-regulating ganglioside synthesis by SK-RC-45, tumor cells were incubated for 5 days in the presence or absence of the inhibitor before isolating and comparing the expression levels of glycosphingolipids from each population by HPTLC, as described in “Materials and Methods.” It is evident that preincubation of SK-RC-45 with PPPP significantly reduced the synthesis and expression of multiple glycosphingolipids discernable as discrete bands on a TLC plate (Fig. 4A), including two with the approximate migration patterns of GD1a and GD1b, one that migrates as GM2 (Fig. 4B) and several that haven’t yet been defined. It was then important to ask whether the recovered expression of gangliosides from the treated cells was attributable to PPPP-mediated inhibition of their synthesis, as has been reported (39), or was rather related to a potential toxicity of the reagent that affected cell viability. Indeed, when SK-RC-45 cells were incubated or not for 5 days with 1 μM PPPP, tumor cell viability was 91.5 and 93.8%, respectively, as assessed by trypan blue at the end of the culture period (Fig. 4C). We were thus confident that any differences detected between the abilities of the PPPP-treated or -untreated tumor cells to induce T-cell apoptosis would be related to the ganglioside expression levels of those two populations.

To assess the role of tumor-derived gangliosides in SK-RC-45-mediated apoptosis of activated T cells, tumor cells pretreated or not with PPPP were cocultivated with activated peripheral blood T lymphocytes for 72 h, at which time target cells were isolated and analyzed for DNA breaks by TUNEL analysis. As compared with control SK-RC-45 cells, which induced 26% of cocultured, activated T lymphocytes to undergo apoptosis, T-cell death was reduced by >50% if ganglioside synthesis by the tumor cell line had been first inhibited by a 5-day pretreatment with PPPP (Fig. 5A). The decreased capacity of PPPP-treated tumor cells to induce T-cell apoptosis was paralleled by their diminished ability to alter the expression levels, subcellular localization, or activities of antiapoptotic and proapoptotic proteins within those lymphocytes. Thus, unlike the untreated RCC cells, which triggered the release of cytochrome c from lymphocyte mitochondria, activated caspases 9 and 3, and inhibited T-cell accumulation of Bcl-2 and Bcl-\textsubscript{XL}, ganglioside-deficient, PPPP-pretreated SK-RC-45 cells induced these changes to a much less extent (Fig. 5B). On the other hand, when PPPP wasn’t added to the SK-RC-45 cells until the tumor cell/T lymphocyte coculture was initiated (i.e., post-treatment), the RCC cells exhibited the approximate potency of untreated tumors in terms of their ability to induce T-cell apoptosis, cytochrome c release, caspase activation, and decreased Bcl-2/Bcl-\textsubscript{XL} expression by the lymphocytes (Fig. 5, A and B).

The differential capacities of ganglioside-expressing and ganglioside-depleted SK-RC-45 populations to mediate proapoptotic changes in normal, activated T cells were reconfirmed in multiple experiments and when Jurkat cells were used as targets. As shown in Fig. 6, there was a full 5-fold difference in the level of Jurkat cell apoptosis induced by ganglioside expressing, as compared with ganglioside-depleted tumor cells. These differences were again reflected in the ability of the respective tumor cell populations to modulate Jurkat cell Bcl-2 and Bcl-\textsubscript{XL} accumulation and cytochrome c localization. Ganglioside-expressing SK-RC-45 stimulated the release of cytochrome c from Jurkat cell mitochondria, lowered the cellular expression levels of the antiapoptotic proteins Bcl-2 and Bcl-\textsubscript{XL} (Fig. 6B), and led to the activation of caspases 9 and 3 (Fig. 6C). In this experiment, caspase 9 and caspase 3 activation was detected by assessing the formation of their active fragments by Western blotting. Ganglioside-depleted RCC
cells caused only minimal mitochondrial release of cytochrome c, significantly less activation of caspases 9 and 3, and had no detectable effects on T cell Bcl-2 and Bcl-XL levels, as assessed by Western analysis (Fig. 6, B and C).

**DISCUSSION**

There are now numerous reports suggesting that tumor-bearing hosts often recognize and initiate T-cell responses to cancer cell antigens: many tumors become heavily infiltrated by T cells (3, 52); lymphocytes with specificity for tumor-specific antigens are detected with enhanced frequency in the peripheral blood of cancer patients (53, 54); and a variety of immune modulators have been demonstrated to stimulate T-cell-mediated tumor regression in murine models of cancer (55, 56). Although antitumor immune responses are demonstrably initiated, however, most appear to be ineffective because tumors typically continue to grow unabated. One explanation for the inadequate host response to cancer is the immunosuppressive nature of the tumor environment (5, 57, 58). In RCC, for example, tumor-infiltrating lymphocytes express only minimal levels of mRNA encoding IL-2 and IFN-γ (58, 59) and <5% of those cells express the activation marker, IL-2Ra (60). The TILs isolated from RCC are also impaired in their proliferative and cytotoxic capacities, and when analyzed by TUNEL, 10–20% of the immune effectors can be clearly identified as being apoptotic (11). The T-cell dysfunction characterizing TILs also extends to the periphery, where delayed-type hypersensitivity to common recall antigens is impaired in 30% of the RCC patients tested, and the lymphocytes from ∼30% of patients are highly susceptible to activation-induced cell death (11).

This study demonstrates that gangliosides play a significant role in the mechanism by which an RCC tumor line stimulates T-cell apoptosis. Our experiments indicate that when incubated in vitro, the RCC cell line SK-RC-45 induces the apoptosis of cocultured normal, activated T cells and Jurkat cells, as measured by TUNEL analysis. Associated with the tumor-induced apoptosis was the SK-RC-45-mediated release of cytochrome c from lymphocyte mitochondria, an appearance of active caspase 9 and caspase 3 in cell lysates, and...
diminished expression of Bcl-2 and Bcl-XL proteins by the cocultured T cells. An important role for RCC gangliosides in these tumor-induced events was suggested by the ability of the ganglioside inhibitor PPPP to reduce SK-RC-45-mediated killing of both Jurkat T cells and normal lymphocytes by at least 50%. This abrogation of RCC-mediated T-cell apoptosis was maximal when tumor cells were preincubated with the ganglioside inhibitor for 5 days before initiating the coculture with lymphocytes, a timeframe consistent with that required for optimally inhibiting ganglioside synthesis with that pharmacological agent (39). Indeed, when ganglioside accumulation by equal numbers of untreated or 5-day PPPP-pretreated SK-RC-45 cells was compared by HPTLC, it was evident that the drug had reduced RCC ganglioside synthesis by 70–80%. This PPPP-mediated reduction in tumor ganglioside synthesis was consistent with the ability of the inhibitor to significantly impede SK-RC-45-mediated cytochrome c release, caspase activation, Bcl-2/Bcl-XL disappearance, and T-cell apoptosis. Interestingly, the expression of several gangliosides was significantly suppressed by the PPPP treatment, including those with the mobility of GD1a, GD1b, and GM2. Specifically, which of these gangliosides or others is mediating the apoptogenic effect is unknown. However, GM2 may be one ganglioside involved in the apoptosis of T cells. The role of this ganglioside in tumor-induced T-cell apoptosis is suggested by preliminary experiments indicating that anti-GM2 antibodies inhibit tumor-mediated T lymphocyte killing by another RCC line, SK-RC-54 (data not shown). Additional experiments are under way to identify the spectrum of RCC-derived gangliosides involved in T-cell killing. This will include the potential involvement of tumor-derived neutral glycosphingolipids, sphyngolipids, and ceramide in the apoptogenic effect.

In the above experiments, PPPP was used to pretreat the tumour cell line and was not present during the final 48-h tumour cell/T-cell cocultures. Thus ganglioside synthesis reinitiated by SK-RC-45 during the coculture may have provided sufficient levels of the glycosphingolipids to account for some of the T-cell killing observed by the PPPP-pretreated tumour line. Conversely, when PPPP was not used as a pretreatment but rather was first added to cultures upon initiation of tumour cell/T-cell coculture (posttreatment), the tumour cells maintained much of their original capacity to induce T-cell apoptosis and the associated proapoptotic molecular changes in cytochrome c release, caspase activation, and altered Bcl-2 and Bcl-XL expression. The modest decrease in apoptogenicity of the PPPP-posttreated tumour cells that is observed likely reflects the component of killing capacity dependent on gangliosides synthesized by the tumor during the final 48 h of incubation.

The notion that SK-RC-45-derived gangliosides contribute to T-cell apoptosis and, hence may participate in immune evasion, is consistent with our previous work, which established that supernatants of explanted RCC tumors either directly stimulated lymphocyte apoptosis or sensitized those cells to activation-induced cell death (20). This work supports those findings by demonstrating that depletion of gangliosides from SK-RC-45 significantly ameliorates the apoptogenicity of that RCC line and extends our knowledge by elucidating some of the molecular mechanisms by which the gangliosides mediate their proapoptotic effects. The fact that, as compared with control tumor cells, PPPP-pretreated SK-RC-45 cells induce less cytochrome c release and caspase activation in target T cells, leading to fewer apoptotic lymphocytes, suggests that ganglioside-mediated activation of the mitochondrial apoptotic pathway is an important mechanism by which the glycosphingolipids act.

There is now accumulating evidence that cell soluble forms of ceramide and some gangliosides may induce apoptosis of various cell types by directly modulating mitochondrial permeability (44, 45). Those molecules cause the accumulation of ROS and the initiation of the mitochondrial permeability transition, leading to cytochrome c release, caspase 9 activation and apoptosis (45, 61). Our findings suggest that gangliosides expressed by the SK-RC-45 line might induce similar changes in T lymphocytes, leading to apoptosis.

The finding that PPPP-pretreatment of SK-RC-45 is able to diminish the inhibitory effect that the SK-RC-45 tumor cell line has on lymphocyte Bcl-XL and Bcl-2 accumulation levels indicates that RCC-derived gangliosides participate in the observed dysregulation of the ant apoptotic proteins. Bcl-XL and Bcl-2 are of key importance in averting Bax-, Bak-, and Bdk-mediated mitochondrial permeability and cytochrome c release (51, 62). Bcl-2 is reported to additionally promote glutathione uptake and thereby augment ROS neutralization (63, 64). Thus, it is possible that by down-regulating both Bcl-2 and Bcl-XL expression levels, RCC-derived gangliosides may promote ROS formation and changes in mitochondrial permeability transition.

We previously showed that several RCC lines, including SK-RC-45, induced apoptosis in activated but not resting T lymphocytes. It should be noted, however, that although SK-RC-45 did not induce DNA breaks in resting T cells, it both altered Bcl-2 and Bcl-XL expression levels in those lymphocytes and caused their externalization of membrane phosphatidyl serine, the latter an early, proapoptotic event. The fact that most TILs infiltrating RCC exhibit an antigen profile characteristic of activated cells (CD45R0+) lends physiological relevance to this observation (12). In previous studies, we demonstrated that RCC cell line-induced apoptosis of both activated T lymphocytes and Jurkat cells could be inhibited 40–50% in the presence of antibodies to FasL (11). Our current results linking SK-RC-45-associated gangliosides to T-cell apoptosis suggest the involvement of multiple tumour-derived products in T-cell killing, which induce apoptosis of T lymphocytes in either an independent or synergistic fashion.

A number of previous reports have provided data to suggest that gangliosides function as soluble modulators of the immune response (28). Gangliosides inhibit multiple steps in the cellular immune response, including antigen processing and presentation (30, 32), lymphocyte proliferation (65), and the generation of cytotoxic responses (66). Especially pertinent in this regard are the reports indicating that tumor cells synthesize and shed aberrantly high levels and altered forms of gangliosides (27–29), which are immunosuppressive in vitro and likely inhibit antitumor immune responses. One study by Ladisch et al. (35) showed that in neuroblastoma patients, the level of shed gangliosides detectable in serum was directly related to the incidence of tumor recurrence and the rapidity of progression. A later analysis by the same laboratory indicated that when assessed by multiple criteria, gangliosides from FBL-3 erythroleukemia cells inhibited tumor-specific secondary proliferative responses and CTL generation in vitro and both primary and secondary antitumor responses in vivo. Ito et al. (67) more recently showed in clinicopathological studies that G1 and G2, two disialogangliosides isolated from the RCC cell line TOS-1, have expression patterns that correlate with the metastatic potential of renal cancer. Although some gangliosides, including GD3, have been shown to stimulate hepatocyte apoptosis, ours is the first report indicating that RCC-induced immunosuppression may be mediated, in part, by its tumor-associated gangliosides. Current research is focused on identifying the specific gangliosides synthesized by RCC and assessing which species are responsible for the observed apoptotic effects. Our results suggest that blocking ganglioside synthesis might augment the efficacy of immunotherapeutic protocols by minimizing tumor-induced T-cell dysfunction and hence promoting more efficacious host-mediated antitumor responses.

4 J. H. Finke, unpublished observation.


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