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TNF-α Induction of GM2 Expression on Renal Cell Carcinomas Promotes T Cell Dysfunction

Gira Raval,* Soumika Biswas,* Patricia Rayman,* Kaushik Biswas,* Gaurisankar Sa,‡ Sankar Ghosh,* Mark Thornton,* Cynthia Hilton,* Tanya Das,‡ Ronald Bukowski,† James Finke,*‡ and Charles S. Tannenbaum2*

Previous studies from our laboratory demonstrated the role of tumor-derived gangliosides as important mediators of T cell apoptosis, and hence, as one mechanism by which tumors evade immune destruction. In this study, we report that TNF-α secreted by infiltrating inflammatory cells and/or genetically modified tumors augments tumor-associated GM2 levels, which leads to T cell death and immune dysfunction. The conversion of weakly apoptogenic renal cell carcinoma (RCC) clones to lines that can induce T cell death requires 3–5 days of TNF-α pretreatment, a time frame paralleling that needed for TNF-α to stimulate GM2 accumulation by SK-RC-45, SK-RC-54, and SK-RC-13. RCC tumor cell lines permanently transfected with the TNF-α transgene are similarly toxic for T lymphocytes, which correlates with their constitutively elevated levels of GM2. TNF-α increases GM2 ganglioside expression by enhancing the mRNA levels encoding its synthetic enzyme, GM2 synthase, as demonstrated by both RT-PCR and Southern analysis. The contribution of GM2 gangliosides to tumor-induced T cell death was supported by the finding that anti-GM2 Abs significantly blocked T cell apoptosis mediated by TNF-α-treated tumor cells, and by the observation that small interfering RNA directed against TNF-α abrogated GM2 synthase expression by TNF-transfected SK-RC-45, diminished its GM2 accumulation, and inhibited its apoptogenicity for T lymphocytes. Our results indicate that TNF-α signaling promotes RCC-induced killing of T cells by stimulating the acquisition of a distinct ganglioside assembly in RCC tumor cells. The Journal of Immunology, 2007, 178: 6642–6652.

The ability to detect tumor-specific T cells in the tumor microenvironment and peripheral blood of many cancer patients strongly implies that immune reactions can be initiated against cancerous tissues (1, 2). However, because tumors most often grow progressively and unabated, it is apparent that the immune responses against them are typically ineffective (2, 3). Numerous tumor immune escape mechanisms have been distinguished to date, and several act by rendering tumor cells immunologically invisible, as follows: tumors can minimize class I and class II Ag expression, and can undergo mutations that inhibit processing and/or presentation of Ags (4). Tumor cells are also known to secrete a variety of immunosuppressive molecules such as TGF-β, IL-10, and PGs, each of which can be inhibitory to infiltrating immune cells (5, 6). Tumor-induced T cell apoptosis is a recently appreciated mechanism of tumor immune evasion, discerned when it was found that an average of 50% (but up to 90%) of T lymphocytes infiltrating the tumor bed appear apoptotic (7) (our unpublished data). Evidence that the tumor or tumor-derived products mediate these apoptogenic effects comes from tumor cell/T cell coculture experiments, which demonstrate that lymphocytes progressively undergo the physiological changes associated with apoptosis with increasing time in coculture (8, 9). Because peripheral blood T cells from many cancer patients are also either apoptotic or exquisitely sensitive to activation-induced cell death, tumors can additionally mediate apoptogenic effects at a distance, by either secreting or sloughing off products that are toxic or inhibitory to the immune system (7). Indeed, we previously reported that conditioned medium from in vivo cultured RCC tumors inhibited NF-κB activation within cocultured T lymphocytes (10), and found that one active component of the supernatants was a ganglioside. Gangliosides are acidic glycosphingolipids present in the outer leaflet of plasma membranes, and are distinguished from another primarily by the number and variety of sugar molecules composing their carbohydrate chains. Gangliosides are ubiquitously expressed, display cell type specificity (11), and are important for their normal roles in signaling, lipid raft structure, and adhesion. Many tumors overexpress and can shed normal or modified forms of specific gangliosides (12), with renal cell carcinomas exhibiting increased levels of GD1a, GM1, and GM2 as compared with normal kidney (13). We determined that such gangliosides play an integral role in the ability of renal tumor cells to induce T cell apoptosis, as when cocultures were performed in the presence of 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPPP),3 an inhibitor of glucosyl transferase, and hence of ganglioside synthesis, tumor-induced cytochrome c release and caspase activation in T cells were inhibited (9).

 highlights the importance of gangliosides in tumor immune evasion and provides evidence for their role in T cell apoptosis.

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3 Abbreviations used in this paper: PPPP, 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol; DAPI, 4',6'-diamidino-2-phenylindole; FasL, Fas ligand; LSA, lipid-bound sialic acid; siRNA, small interfering RNA; VHL, von Hippel-Lindau.

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We now show that GM2 production by RCC directly correlates with the ability of those tumors to mediate T cell apoptosis, and demonstrate that TNF-α induces the synthesis of this ganglioside, regardless of whether TNF-α is made by the tumor cells themselves, or is administered to the tumor cells exogenously. The in vivo relevance of this observation stems from our determination that 19 of the 24 fresh RCC tumors we assessed were GM2 positive (14). Although TNF-α is most often considered in the context of its essential role in both innate and adaptive immune responses (15), it was originally described as an endotoxin-induced, macrophage-derived protein capable of mediating tumor necrosis (16). It is now found that TNF-α produced at pathophysiological concentrations actually promotes tumor growth (17), suggesting that the cytokine may have different effects depending on the tumor type to which it is administered. Indeed, the effect of TNF-α on renal cancer cells is dependent on whether the tumor expresses the wild-type or mutant form of the von Hippel-Lindau (VHL) gene: wild-type tumors are typically susceptible to the toxicity of TNF-α, although many VHL mutants are both resistant to and themselves produce the cytokine (18, 19). The following studies were initiated upon determining that TNF-α-producing RCC lines were significantly more apoptogenic for T cells than were nonproducers, and that nonapoptogenic lines could be converted to killers if first stimulated with TNF-α. The findings support the notion that TNF-α can promote tumor immune escape by stimulating the production of apoptogenic/immunosuppressive gangliosides by RCC.

Materials and Methods

Reagents

Ficoll-Hypaque was purchased from Amersham Biosciences. Anti-human T cell enrichment mixture was obtained from StemCell Technologies. Purified bovine brain-derived gangliosides GM3, GM1, GM2, GD1a, and GD2 were purchased from Matreya, and GD3 was purchased from Calbiochem. A hamster anti-GM2 mAb (DMF10.167.4) was a gift from K. Rock (University of Massachusetts, Worcester, MA). Mouse anti-human GD1a was purchased from Seikagaku Kogyo, and both mouse anti-human GD3 and mouse anti-human GD2 were purchased from BD Pharmingen. Purified hamster IgG was from Santa Cruz Biotechnology, and annexin V was obtained from BD Biosciences. Anti-TNF-α Ab was purchased from Santa Cruz Biotechnology. FITC-conjugated anti-hamster Ig was purchased from BD Biosciences, and FITC-conjugated anti-rabbit Ig was purchased from Molecular Probes. An apoptotic anti-Fas Ab was purchased from Upstate Biotechnology, and was used at a concentration of 2.0 μg/ml to induce apoptosis of T cells through the Fas receptor. A TNF ELISA kit was purchased from R&D Systems, and was used according to manufacturer’s instructions to assess TNF production (ng/ml/10⁶ cells) by the TNF-transfected SK-RC-45 RCC clones. HPLC grade acetoneitrile, methanol, analytical grade chloroform, isopropanol, diisopropyl ether, n-butylacetate, and n-butanol were obtained from Fisher Scientific. Sialic acid (α-acetylated-neuraminic acid) phosphatidic acid, and reserpinol were purchased from Sigma-Aldrich. FBS was purchased from HyClone. Glutamine, gentamicin, MEM sodium pyruvate solution, and MEM nonessential amino acid solutions were obtained from Invitrogen Life Technologies.

RCC cell culture

The well-established RCC lines SK-RC-45, SK-RC-54, SK-RC-13, and SK-RC26b were a gift from N. Bander (New York Hospital, Cornell University Medical College, New York, NY). These cells were maintained in complete RPMI medium, as described previously (9). The small interfering RNA (siRNA) transfection was performed using the lipofectamine 2000 reagent on 75–80% confluent SK-RC-26 cells grown in 35-mm dishes at 37°C for at least 2 h in 800 μl of OptiMEM medium. Briefly, cells were treated for 7 h with 200–400 pmol siRNA directed against human TNF-α, and then were brought up to 2 ml in OptiMEM and incubated for an additional 24 h. After additional 12 h in RPMI 1640 + 10% FBS, cells were transfected a second time, as described above, to maintain TNF-α protein levels at their reduced levels for the duration of the experiment.

Isolation of T lymphocytes

Peripheral blood was obtained from healthy volunteers with informed consent (IRB 1382) and then centrifuged over a Ficoll-Hypaque density gradient (Amersham Biosciences) to obtain total leukocytes. T cells were purified by negative magnetic selection using microbeads coated with Ab against CD14 (macrophages), CD16 (NK cells), CD19 (B cells), CD56 (NK cells), and glycophorin A (RBCs) (StemCell Technologies). The T cell isolation procedure yielded cells that were >95% positive for CD3, as defined by immunocytometry.

Assessment of T death following coculture with tumor lines

SK-RC cell lines were seeded in 100-mm tissue culture dishes and allowed to reach confluence (~3.0 × 10⁶ cells). T cells were resuspended to a concentration of 1 × 10⁶ cells/ml in complete RPMI 1640 before being added to the 100-mm dishes containing SK-RC cells at a 3:1 tumor:T cell ratio. T cells were removed from the SK-RC monolayers after 72 h by gentle washing. T cell death was assessed by trypan blue. T cell apoptosis was determined by TUNEL using the APO-BRDU system (Phoenix Flow Systems). The percentage of apoptotic T cells present in each cell population was determined using quadrant analysis software (LYSIS II; BD Biosciences).

Plasmid construction and TNF-α transfection

cDNA encoding TNF-α was generated by performing RT-PCR on RNA isolated from anti-CD3/anti-CD28-stimulated T cells, and was cloned into the HindIII and Xba sites of pCDNA3. A recombinant plasmid lacking mutations was selected by DNA sequence analysis, and was shown by transient transfection and Western analysis to direct TNF-α protein synthesis. Plasmids were transfected into SK-RC-45 cells using the Amaxa system Kit V (Amaxa Biosystems), with the Amaxa nucleoporator set to transient transfection and Western analysis to direct TNF-α protein synthesis.

Lipid-bound sialic acid (LSA) assay

The assay to measure total plasma ganglioside (expressed as LSA) was performed, as previously reported (20). Briefly, 50 μl of plasma was extracted with a 2:1 ratio of ice cold chloroform-methanol. The lipid extract was subsequently separated with 0.5 ml of cold distilled water, and the LSA content of the aqueous phase was quantified using the reserpinol-HCl reagent, by comparing the absorption at 580 nm with the standard curve generated using known amounts of free sialic acid (α-acetylated-neuraminic acid) (20).

Isolation and HPLC analysis of gangliosides

Tumor cell lines were grown to 80% confluency in complete RPMI 1640 before being collected and washed twice in PBS. Gangliosides were isolated from the cell pellets essentially as described before (21, 22). The composition of water-soluble gangliosides isolated from the RCC lines was determined by HPLC using a Beckman Coulter HPLC system. A normal-phase Varian Microsorb-NH₃ column (25 cm, 100 Å) was used as the stationary phase. Ganglioside separation was conducted with a gradient of the following solvent mixtures, used as the mobile phase: solvent A, acetoni-­trile-20 mM phosphate buffer (pH 5.6) (50:50); solvent B, acetoni-­trile-5 mM phosphate buffer (pH 5.6); solvent C, 83:17; solvent D, 100% solvent B. The gradient elution program was the same as described earlier (23). The flow rate was maintained at 1 ml/min, and the elution profile was monitored by flow-through detection of UV absorbance at 197 nm. The retention times of gangliosides isolated from RCC were compared with the retention times of purified bovine brain-derived gangliosides.

Measurement of gangliosides by ELISA

To quantify the TNF-mediated change in SK-RC-45-associated gangliosides for which specific commercial Abs were available, ELISAs were set up, as described previously (24). Briefly, standard bovine brain-derived gangliosides derived from tumor cell lines or tumor tissue were resuspended in 1:1 solution of CHCl₃/MeOH at 1 mg/ml, and appropriate aliquots of these solutions were added to individual wells containing 100 μl of MeOH. Gangliosides were dried onto the plates by an overnight incubation at 37°C, at which time wells were treated for 2 h at 37°C with 200 μl of blocking buffer (1% BSA in PBS). Blocking buffer was then removed and replaced with 200 μl of Ab at 1 μg/ml in PBS. One hour later, wells were washed with 0.5% Tween 20 in PBS and dried by inversion over a piece of blotting paper. Two hundred microliters of a peroxidase-conjugated secondary Ab diluted 1/1000 in 1%
Results

**TFN-α enhances the ability of RCC lines to induce apoptosis in resting T cells**

Previous work from our laboratory demonstrated that the RCC cell line SK-RC-45 induced the apoptosis of anti-CD3/CD28-activated T cells by a mechanism involving tumor-derived gangliosides, because the killing of cocultured lymphocytes was largely abrogated when the tumor cells were pretreated with the ganglioside synthase inhibitor PPPP. Although bulk SK-RC-45 was incapable of mediating the apoptosis of resting T cells to a significant extent, the ability of an isolated, SK-RC-45 subclone to do so correlated with its elevated TNF-α production levels. Thus, to determine whether exogenously administered TNF-α could render bulk, non-apoptogenic SK-RC-45 cells toxic for resting T lymphocytes, the RCC line was pretreated with 250 ng/ml rTNF-α for 5 days before initiating coinoculation. Unlike a 1- or 2-day treatment with TNF-α, which had little or no impact on the ability of SK-RC-45 to kill resting T cells over a 72-h time frame (data not shown), a 3- to 5-day pretreatment with the cytokine converted the weakly apoptogenic line into a population able to kill 32% of the cocultured lymphocytes, as measured by trypan blue positivity (Fig. 1A, left), and to induce an average of 32% to an apoptotic state, which had little or no impact on the ability of SK-RC-45 to kill resting T cells (data not shown).

Loss of function mutations in the VHL tumor suppressor protein are common in clear cell RCC (25), with ~50% of patients testing positive for those genetic alterations (26). Although the wild-type VHL protein was known to modulate the expression of multiple gene products (19), it was only recently shown that TNF-α mRNA more avidly associates with polyosomes in RCC cells harboring the mutation (27), explaining why many RCC tumors, but not normal kidney cells, synthesize that cytokine constitutively. Because immunohistological examination of many explanted RCC tumors

BSA/PBS was then added to the appropriate wells, and the plate was incubated at 37°C for 30 min. Wells were again washed five to six times, as described above, developed according to manufacturer’s instructions (BD OptEIA; BD Biosciences), and measured using a PerkinElmer luminometer set to detect luminescence at 450/570 nm.

**Immunofluorescence**

Tumor lines were immunostained to assess GM2 and TNF-α expression levels. Control and TNF-α-stimulated or transfected SK-RC-45 cells were fixed in paraformaldehyde and permeabilized with Triton X-100 (Sigma-Aldrich). Cells were then washed with PBS, blocked with 1% BSA, and incubated overnight at 4°C with either anti-GM2 or anti-TNF-α Abs. After another wash, the cells were incubated with the appropriate, labeled secondary Abs (Molecular Probes) and counterstained with 4,6-diamidino-2-phenylindole (DAPI) to visualize the nuclei.

**RT-PCR analysis of GM2 synthase mRNA levels**

Total RNA was isolated from SK-RC-45 cells using the RNAeasy minikit (Qiagen), following the manufacturer’s protocol. For RT-PCR analysis of GM2 synthase (β4GalNAc-T) and GAPDH mRNA, the SuperScript First-Strand Synthesis System (Ambion) was used to synthesize the respective cDNAs. PCR was conducted using 5′-ACCTCCGGCAACTTGTCAGTCC-3′ as the sense primer and 5′-ATCCGCCTACGGGCCTTCATCACCC-3′ as the antisense primer, which gave a 457-bp fragment of GM2 synthase. GAPDH primers were as follows: 5′-ACCTGGCCAAGGCTATCCT-3′ (sense) and 5′-TCCACACCTGTTGCTGTA-3′ (antisense), giving an expected product of 506 bp. The reaction was conducted for 35 cycles, using the following parameters: 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. Samples were electrophoresed on a 2.0% agarose gel and visualized by ethidium bromide staining.

**FIGURE 1.** SK-RC-45 cells either pretreated with TNF-α for 5 days or transfected with cDNA encoding TNF-α are much more effective at inducing apoptosis of resting T cells than are untreated tumor cells. A, Left, Peripheral blood T cells were cocultured for 48 h with either medium, TNF-α (250 ng/ml), SK-RC-45, or SK-RC-45 cells pretreated with TNF-α (250 ng/ml) for 5 days, before assessing lymphocyte viability. Cell viability was assessed by staining T cells with trypan blue. Data presented are the mean (±SD) of six different experiments. A, Right, Using the same culture conditions as described for the left panel, tumor-induced T cell apoptosis was determined by TUNEL analysis. Data presented are the mean (±SD) of three different experiments. B, Resting T cells were cultured for 72 h either alone or with TNF-α, wild-type SK-RC-45, or two individual SK-RC-45 clones expressing the TNF-α transgene, at which time the lymphocytes were removed and assessed by trypan blue staining for viability. The data presented represent the mean (±SD) of three experiments.
reveals that a large percentage of the infiltrating T cells can be apoptotic, we asked whether transfection of cDNA encoding TNF-α into the comparatively nonapoptogenic, essentially TNF-α-negative SK-RC-45 cells could enhance the line’s capacity to kill resting T lymphocytes. G418-resistant SK-RC-45 clones transfected with a TNF-α-encoding plasmid were isolated, as described in Materials and Methods, and were screened for TNF-α production using multiple techniques. Immunofluorescence studies revealed that two G418-resistant clones (clones 24 and 13) expressed significantly more TNF-α intracellularly than did the parental SK-RC-45 cells (data not shown), findings that correlated with ELISA results indicating that the same two clones secreted ~100 times more TNF than controls (see below). It was also apparent that the TNF-α-producing tumor cells were much more toxic for cocultured resting lymphocytes than were the control tumor cells. That is, as compared with the untransfected SK-RC-45, or with exogenously administered rTNF-α alone, which each induced essentially background levels of T cell death (6.2 and 10%, respectively), TNF-α-transfected clones 13 and 24 exhibited almost equivalent, enhanced apoptogenic effects, inducing ~44% of the cocultured resting T cells to die (Fig. 1B). It was thus clear that the TNF-α transgene was capable of converting a fundamentally nonapoptogenic tumor line into one capable of mediating T cell killing.

**FIGURE 2.** Overexpression of the TNF-α transgene in the SK-RC-45 line induces augmented ganglioside expression. **A**, TNF-α-induced ganglioside expression was assessed by comparing the total LSA from wild-type SK-RC-45 cells and TNF-α-overexpressing clone 13 cells. Total gangliosides were extracted from 1 million control and TNF-α-transfected SK-RC-45 cells with (2:1) CHCl3:MeOH, which were then subjected to sialic acid analysis using the resorcinol-HCl reagent described in Materials and Methods. Clone 13 showed a significant (p < 0.006) increase in total ganglioside content as compared with parental cells. The data presented represent the mean (±SD) of three experiments. **B**, Gangliosides isolated from control (left panel) and TNF-α-transfected SK-RC-45 cells (clone 13) (right panel) were subjected to HPLC fractionation, as described in Materials and Methods. The identities of individual gangliosides (monitored at 197 nm) isolated from wild-type and TNF-α-transfected SK-RC-45 cells were determined by comparing their retention times with those of purified bovine brain-derived gangliosides (monitored at 197 nm) fractionated on the same column. Similar results were obtained in three HPLC runs. **C**, Tumor gangliosides were isolated, dissolved in CHCl3:MeOH at the appropriate concentrations, and then adhered to the wells of a 96-well plate, as described in Materials and Methods. After the wells were blocked with 1% BSA in PBS, and treated with both primary anti-ganglioside Abs and appropriate peroxidase-conjugated secondary Abs, the wells were exposed to the color development solution for 15 min in the dark before being fixed and read on a PerkinElmer luminometer set to read luminescence at 450/570 nm. GM2 data are the mean (±SEM) of two separate experiments. **D**, Wild-type SK-RC-45, clone 13, and clone 24 cells were grown on coverslips and then subjected to immunofluorescence staining, as described in Materials and Methods, using Ab specific for GM2. Staining with IgG was performed as a negative control, and gave completely negative results (data not shown). Top panels, Bright field. Bottom panels, Green fluorescent staining for GM2, with nuclei revealed by DAPI staining.
gangliosides, and sialic acid are gangliosides (29, 30). Indeed, LSA analysis revealed the LSA content of both cell types, because most lipids bound to sialic acid are gangliosides (29, 30). We compared the time points at which those features were induced. Replicate cultures of SK-RC-45 cells, growing in tissue culture plates, were cultured in fresh medium. Supernatants were subsequently assessed by ELISA for TNF-α-induced GM2 accumulation. As depicted in Fig. 4A, SK-RC-45 pretreated with TNF-α for 1 day expressed only low levels of GM2, and were not able to kill resting T cells (Fig. 4B). By the third day of TNF-α treatment, however, substantial GM2 expression was detected on the SK-RC-45 tumors and results are reported as ng of TNF-α produced per one million cells in a 24-h period. Data presented are the mean (±SD) of three different experiments.

**FIGURE 3.** TNF-α stimulation of GM2 synthase mRNA accumulation mediates the observed increase in GM2 expression. A, RNA preparations isolated from wild-type SK-RC-45 and three stable, TNF-α-transfected clones were subjected to RT-PCR using 5′ and 3′ primers for GM2 synthase. A RT-PCR using the primers for GAPDH served as a control for RNA levels in each sample. RT-PCR products were generated by 30 cycles of amplification, and were subjected to electrophoresis on a 2.0% agarose gel, and visualized by ethidium bromide staining. B, Each of the clones described in A producing differing amounts of TNF-α were cultured in 100-mm dishes until confluent, and were then incubated an additional 48 h in fresh medium. Supernatants were subsequently assessed by ELISA for TNF-α synthesis, and results are reported as ng of TNF-α produced per one million cells in a 24-h period. Data presented are the mean (±SD) of three different experiments.

To directly test the participation of TNF-α-induced GM2 accumulation in the apoptosis of T cells mediated by TNF-α, SK-RC-45 were pretreated with TNF-α (27 ng/10⁶ cells/24 h) than clones 13 (293 ng/10⁶ cells/24 h) and 24 (236 ng/10⁶ cells/24 h) also expressed commensurately lower levels of GM2 synthase mRNA (Fig. 3), but did have higher levels than seen in the untransfected control. These findings suggest that TNF-α may promote GM2 expression in SK-RC-45 cells by increasing the level of the key enzyme for GM2 synthesis. It should be noted that whereas surface Fas ligand (FasL) was also highly induced in the TNF-producing clones, as assessed by both Western analysis and confocal microscopy (data not shown), its enhanced expression was essentially irrelevant to these studies: even high concentrations of anti-Fas Abs (2 μg/ml) did not mediate apoptosis of the unstimulated, peripheral blood T cells used in the experiments reported in this study (data not shown).

The TNF-mediated, incremental accumulation of GM2 by SK-RC-45 over time correlates with the cell line’s TNF-augmented apoptogenicity

To assess the possibility that it is the TNF-α-mediated increase in GM2 expression that renders SK-RC-45 highly apoptogenic, we compared the time points at which those features were induced. Replicate cultures of SK-RC-45 cells, growing in tissue culture dishes and on coverslips, were incubated or not with 250 ng/ml rTNF-α for multiple time periods, and at each point the cultures were evaluated for their capacity to mediate the apoptosis of coincubated resting T cells, and the slides were appraised immunohistochemically for TNF-α-induced GM2 accumulation. As depicted in Fig. 4A, SK-RC-45 pretreated with TNF-α for 1 day expressed only low levels of GM2, and were not able to kill resting T cells (Fig. 4B). By the third day of TNF-α treatment, however, substantial GM2 expression was detected on the SK-RC-45 cells (Fig. 4A), and the tumors had acquired the capacity to induce T cell death to levels three times higher than background (Fig. 4B). Both TNF-α-induced GM2 accumulation and SK-RC-45 apoptogenicity reached their highest levels by 5 days of treatment, at which time the tumor line killed 33% of the coincubated lymphocytes (Fig. 4B). These experiments thus suggested that the time frame necessary for TNF-α to enhance SK-RC-45 killing coincided well with the TNF-α-mediated accumulation of GM2.

TNF-α-enhanced apoptogenicity of SK-RC-45 is partially blocked by anti-GM2 Abs

To directly test the participation of TNF-α-induced GM2 accumulation in the apoptosis of T cells mediated by TNF-α-stimulated SK-RC-45, the parental tumor line was pretreated or not with TNF-α for 5 days before coculturing the cells with T lymphocytes for 72 h in the presence or absence of Abs to GM2. As anticipated, the SK-RC-45 cells pretreated with rTNF-α induced significantly more T cell killing than did unstimulated SK-RC-45 (32 vs 8%; Fig. 4C). When the cocultures took place in the presence of Abs to GM2, the apoptogenicity of the TNF-α-treated SK-RC-45 cells...
was inhibited by ~50%, although isotype control Abs were without effect (Fig. 4C). The ability of anti-GM2 Abs to inhibit T cell killing by TNF-α-stimulated SK-RC-45 cells indicates that this TNF-α-inducible ganglioside is directly involved in the apoptogenic activity of the tumor cells.

**TNF-α-inducible GM2 expression and apoptogenicity are common to multiple RCC lines**

The correlation between GM2 expression and SK-RC-45 apoptogenicity suggested the possibility that other RCC lines too might kill T cells by a mechanism involving TNF-α-augmented GM2 levels. As illustrated by the graph in Fig. 5, unstimulated RCC line SK-RC-54 was ineffective at killing cocultured T cells, but like SK-RC-45, when treated for 5 days with rTNF-α, both its GM2 accumulation (Fig. 5A) and apoptogenicity for T cells (Fig. 5B) were dramatically increased as follows: the percentage of T cells killed by SK-RC-54 rose from 4 (essentially background levels) to 23% following treatment with rTNF-α. Similar increases in both GM2 expression and apoptogenicity were seen when RCC line SK-RC-13 was stimulated with rTNF-α (data not shown). The ability of TNF to augment GM2 levels in RCC cells is not limited to long-term RCC lines. Interestingly, three distinct patterns of GM2 expression were observed when cells isolated from fresh RCC tumors were digested into single cell suspensions, plated onto coverslips, and then treated or not for 4 days with 250 ng/ml TNF. Some tumors expressed highly elevated levels of GM2 constitutively. Others expressed low levels of GM2 constitutively, and were induced to elevated GM2 synthesis by in vitro treatment with TNF. Interestingly, cells isolated from the single normal adjacent kidney sample neither expressed GM2 constitutively, nor were induced to do so following exposure to TNF. Thus, based on our in vitro data, the ability of rTNF-α to increase both the GM2 levels and apoptogenicity of multiple RCC lines, by a mechanism that is reversible by anti-GM2 Abs, suggests that TNF-α-induced ganglioside expression is a general mechanism by which RCC tumors can mediate T cell apoptosis.
SK-RC-26b is constitutively apoptogenic by virtue of its constitutive elaboration of TNF-α and GM2; treatment of the RCC line with siRNA to TNF-α 3 days before T cell coculture experiments inhibits SK-RC-26b-mediated T cell killing.

Some RCC lines such as SK-RC-26b are characterized by already elevated GM2 levels, and do not require additional rTNF-α treatments to promote high expression of that ganglioside (Fig. 6A, bottom). Such tumor lines also constitutively kill resting T cells in

FIGURE 5. TNF-α-inducible GM2 expression and apoptogenicity are common to multiple RCC lines. The nonapoptogenic SK-RC-54 RCC cell line was treated or not for 5 days with 250 ng/ml TNF-α before being subjected to immunostaining with anti-GM2 Abs, or to coculture studies with resting T cells. A, Immunostaining of SK-RC-54 cells treated or not with rTNF-α for 5 days. Top panels: Bright field (×40 magnification). Bottom panels: Immunostaining with anti-GM2 Abs, demonstrating elevated expression of the ganglioside by the TNF-α-treated cells. B, Control and TNF-α-treated SK-RC 54 cells were coincubated with resting T cells for 72 h before assessing the isolated lymphocytes by trypan blue exclusion for tumor-induced T cell killing. Data presented are representative of three different experiments.

FIGURE 6. An RCC cell line constitutively synthesizing elevated levels of GM2 also constitutively synthesizes TNF, and is constitutively apoptogenic. A, SK-RC-45 (top) and SK-RC-26b (bottom) were subjected to immunostaining with Abs directed against GM2, as described in Materials and Methods, to assess their respective, constitutive expression levels of that ganglioside. B, Resting T cells were cultured for 72 h either alone, with SK-RC-45, or with SK-RC-26b, at which time the lymphocytes were removed and assessed by trypan blue staining for viability. Data presented are the mean (±SD) of three different experiments. C, Immunostaining with anti-TNF-α was performed on SK-RC-26b, as described in Materials and Methods, demonstrating that the RCC line was constitutively expressing the cytokine.

FIGURE 7. siRNA targeting constitutively synthesized TNF-α in SK-RC-26b turns off TNF-α and GM2 expression by that cell line, and inhibits its ability to kill resting T cells. A, SK-RC-26b was treated with siRNA-targeting TNF-α, as described in Materials and Methods, 3 days and then again 1 day before initiating a 72-h tumor cell/T cell coculture experiment. Immunostaining with Abs to TNF-α and to GM2 was performed on untreated SK-RC-26b cells and 24 h following the second siRNA treatment, to assess the ability of the siRNA to turn off both TNF-α and GM2 expression by the RCC cell line. Staining with IgG was performed as a negative control, and gave completely negative results (data not shown). B, Replicate plates of SK-RC-26b, treated or not with siRNA-targeting TNF-α, as described above, were cocultured with resting T cells for 72 h before removing the lymphocytes and assessing them by annexin V/7-aminoactinomycin D staining for tumor-induced killing. Data presented are the mean (±SEM) of two different experiments.
coculture experiments, as demonstrated in Fig. 6B: as compared with the bulk population of SK-RC-45, which in its unstimulated state kills an average of 12% of cocultured T cells vs a background of 8%, untreated SK-RC-26b kills an average of 32% of coincubated T lymphocytes. Given the experiments described above associating TNF-α-induced GM2 expression with TNF-α-induced apoptogenicity, one might predict that the constitutive apoptogenicity of clone 26b is based on the line’s constitutive synthesis of TNF-α. Indeed, immunostaining with Abs to that cytokine suggests that SK-RC-26b does in fact constitutively synthesize abundant levels of TNF-α (Fig. 6C). To determine whether there is a linkage between SK-RC-26b apoptogenicity and the tumor’s constitutive synthesis of TNF-α and GM2, that cell line was treated with siRNA-targeting TNF-α mRNA 3 days and then again 1 day before initiating 72-h tumor cell/T cell cocultures. Immunostaining of treated, replicate cultures revealed that by the time of 8%, untreated SK-RC-26b kills an average of 32% of coincubated T cells vs a background state kills an average of 12% of cocultured T cells vs a background of 8%, untreated SK-RC-26b kills an average of 32% of coincubated T lymphocytes. Given the experiments described above associating TNF-α-induced GM2 expression with TNF-α-induced apoptogenicity, one might predict that the constitutive apoptogenicity of clone 26b is based on the line’s constitutive synthesis of TNF-α. Indeed, immunostaining with Abs to that cytokine suggests that SK-RC-26b does in fact constitutively synthesize abundant levels of TNF-α (Fig. 6C). To determine whether there is a linkage between SK-RC-26b apoptogenicity and the tumor’s constitutive synthesis of TNF-α and GM2, that cell line was treated with siRNA-targeting TNF-α mRNA 3 days and then again 1 day before initiating 72-h tumor cell/T cell cocultures. Immunostaining of treated, replicate cultures revealed that by the time.

Discussion

A number of studies suggest that tumor products present within and secreted from the tumor microenvironment can induce T cell apoptosis, partially explaining the immune dysfunction characterizing cancer patients (31). When RCC tumors are excised and assessed by in situ TUNEL analysis, many are found to contain apoptotic T cells: flow cytometry indicates that >30% of the tumor-infiltrating T cells, and sometimes as many as 100%, are annexin V/7-aminoactinomycin D positive (A. Richmond, P. Rayman, P. Elson, R. Bukowski, A. Novick, and J. Finke, manuscript in preparation). These results are not unique to renal cell carcinoma, because T cells isolated from the bloodstream and tumor microenvironments of patients with glioblastoma, colorectal cancer, and squamous cell carcinoma of the head and neck are also found to be either apoptotic or highly sensitive to activation-induced cell death (32–34).

A variety of mechanisms has been proposed to explain the predisposition of cancer patients’ T cells to apoptosis. Histologically diverse tumors may express death ligands that mediate the apoptosis of tumor-infiltrating lymphocytes (35, 36), a notion supported by the immunohistological colocalization of apoptotic T lymphocytes with FasL-, TRAIL-, or CD70-bearing malignant cells (37–39). Others maintain that it is not the death ligands expressed by the tumor that kill infiltrating T cells, but rather it is the FasL expressed on the lymphocytes themselves that reciprocally induce each other to apoptosis when the cells come into close contact (40). Such fratricide would be more characteristic of activated T cells expressing elevated levels of FasL, a phenomenon most apt to occur in cancer patients following T cell recognition of tumor Ags (40).

Previous studies from our group established that soluble tumor products also play a role in mediating T cell apoptosis (41). Supernatants collected from in vitro, explanted RCC tumors induced T cell apoptosis within 72 h of coincubation, and experimental evidence suggested that tumor-derived gangliosides were an active, apoptogenic component of the tumor-conditioned medium, as follows: neuraminidase abrogated the effect (10), gangliosides extracted from the supernatants demonstrated equivalent apoptogenicity (9, 10), and the ganglioside synthesis inhibitor PIPP reduced SK-RC-45-mediated killing of both Jurkat cells and activated T lymphocytes by at least 50% (9). Such glycosphingolipids are overexpressed by a variety of tumor types, and appear to mediate their apoptotic effects by both inhibiting NF-κB (42) and acting directly on target cell mitochondria to induce cytochrome c release and subsequent caspase activation (43).

In this study, we demonstrate that RCC tumor cells are rendered highly apoptogenic by a mechanism involving the TNF-α-enhanced production of GM2. Multiple experiments presented in this study link RCC-derived GM2 to tumor apoptogenicity, and GM2 accumulation by tumors to the paracrine or autocrine TNF-α stimulation of renal carcinoma cells. Whether administered exogenously or via a transgene, TNF-α-stimulated RCC cells overexpressed GM2 and induced cocultured, resting T cells to TUNEL positivity and death. There may be several explanations for why only a subpopulation of the T cells exposed to TNF-stimulated SK-RC-45 tumor cells became apoptotic during coculture. First, all of the experiments reported in this work used unstimulated peripheral blood T cells, and we find them to be less susceptible to tumor-induced apoptosis than freshly activated T cells. Thus, the 33% of lymphocytes rendered apoptotic by the TNF-stimulated tumor cells may represent a more activated T lymphocyte component of the population. Also, because the 3-day time point was arbitrarily chosen to be the duration of coculture, it may be that more lymphocytes would have been killed if the coincubations were terminated at a later time point. It should be noted that TNF was also found to induce elevated levels of tumor-associated FasL. However, in multiple experiments, we, like others, have determined that freshly isolated peripheral blood T cells are resistant to Fas-mediated apoptosis (44), suggesting that the TNF-induced FasL expression by SK-RC-45 is irrelevant to the tumor-induced death reported in this study.

There is precedence to the notion that TNF-α induces ganglioside expression, as follows: incubation of normal melanocytes with TNF-α increased their production of both GM3 and GD3 (45), and pancreatic islet cells similarly showed increased ganglioside expression following TNF-α treatment (46). Additionally, studies with TNFR1-deficient mice revealed the importance of TNF-α signaling for maintaining expression of select gangliosides in normal tissues, such as lung, muscle, thymus, and spleen (28). When compared with wild-type mice, TNFRI-deficient animals demonstrated decreased expression of GM3-Neu5Ac, GM3-Neu5Gc, and GM1b, with a corresponding increase in the neo-lacto series of gangliosides (28).

The initial observation leading to our studies was the finding that an RCC isolate exhibiting unusually elevated apoptogenicity for resting T cells also synthesized high levels of TNF-α. Because TNF was reported to stimulate ganglioside production in normal cells (28, 45), this suggested the possibility that a newly induced RCC ganglioside might be the mediator of this lymphocyte toxicity, especially because we had already demonstrated a role for gangliosides in tumor-induced T cell apoptosis (9, 10). Indeed, lipid analysis of TNF-α-transfected SK-RC-45 tumor cells demonstrated a 2-fold higher sialic acid content than detectable in the untransfected parental line. Because most sialic acid-containing lipids are gangliosides (20), this result supported the notion that
ganglioside production was in fact enhanced in the TNF-α-producing clone. HPLC analysis of gangliosides isolated from parental and TNF-α-transfected SK-RC-45 cells confirmed this view, because the accumulation of several gangliosides, including GM2, was enhanced in the TNF-α-producing clone. ELISA studies confirmed that TNF increased GM2 accumulation in a TNF concentration-dependent manner, seemingly down-regulated GD2 expression, and had essentially no effect on GD3 expression. Immunostaining verified the HPLC and ELISA findings for GM2: unlike the untransfected RCC cells, the two TNF-α-transfected lines both stained strongly for TNF-α and GM2. RT-PCR analysis provided insight into the mechanism by which TNF-α modulated GM2 levels: there was little GM2 synthase mRNA amplified from parental SK-RC-45/RNA, and only slightly more when RNA from a low TNF-α-expressing clone was used as a template, although the band intensities were dramatically increased when the RNA amplified was derived from the high TNF-α-producing RCC clones 13 and 24. Thus, TNF-induced GM2 synthase expression is the likely mechanism by which GM2 levels become elevated in TNF-stimulated SK-RC-45 cells.

Multiple experimental findings likewise linked the TNF-α-stimulated production of tumor-associated GM2 with the acquired ability of the cancer cells to induce T cell apoptosis. First, there was a close correlation between the time it took TNF-α to stimulate both effects, as follows: TNF-α detectably enhanced GM2 accumulation by day 3 of treatment, and increased it to maximal levels by day 5. This corresponded well to the time frame over which TNF-α differentially induced apoptosis. Consistent with these findings was the fact that it was the TNF-α-transfected SK-RC-45 clones synthesizing the most cytokine, which also accumulated the most GM2 and were most apoptogenic. Moreover, this apoptogenicity could be inhibited if the TNF-α-transfected SK-RC-45/T cell cocultures were treated with Abs to GM2 during the 72 h of coculture.

We also find that tumor supernatants containing GM2, as determined by HPLC and mass spectrophotometric analysis, can similarly induce T cell apoptosis, as can ganglioside preparations purified from tumor supernatants. Abs to GM2, but not to other gangliosides tested, inhibit the apoptotic effects of these tumor supernatants and isolated gangliosides by >50%, indicating that, indeed, it is the soluble GM2 in these supernatants that mediate at least some of the activity (G. Sa, S. Ghosh, P. Rayman, C. Tannenbaum, A. Novick, R. Bukowski, and J. Finke, manuscript in preparation). In contrast, GM2-containing supernatants have no adverse effects on non-GM2-producing RCC tumor lines.

Very preliminary studies from our laboratory suggest that at least a subset of the T cells killed in cocultures with GM2-producing tumors are those lymphocytes that incorporate the ganglioside into their cell membranes and endocytic vesicles (C. Hilston, K. Biswas, J. Finke, and C. Tannenbaum, manuscript in preparation). This observation is supported by previous reports indicating that under certain stimulatory conditions, gangliosides are transported in endocytic vesicles from the cell membrane to the mitochondria, where, in glutathione-depleted cells, they can induce reactive oxygen species, mitochondrial permeability, cytochrome c release, caspase 9 activation, and apoptosis (47). Interestingly, a number of T cells isolated from the peripheral blood of many RCC patients, but not from normal controls, also have membrane- and cell-associated GM2, and in most cases are annexin V positive (S. Biswas, A. Richmond, K. Biswas, S. Ghosh, C. Tannenbaum, R. Bukowski, A. Novick, and J. Finke, manuscript in preparation). This relationship appears to extend to other leukocytes as well: although viable if isolated from normal controls, we find that NK cells and B cells isolated from many RCC patients contain cell-associated GM2, and 50% are annexin V positive when assessed by FACS analysis (manuscript in preparation). Thus, in addition to T lymphocytes, even other cells of the immune system may be susceptible to the deleterious effects of tumor gangliosides.

The cellular source of TNF-α in renal tumors most likely includes macrophages, activated T cells, and tumor cells. In a recent analysis of 83 RCC patients, TNF-α was produced by tumor-associated macrophages, and a positive correlation between TNF-α production and tumor size was observed in stage T1 RCC patients (48). RCC tumors have also been reported to produce TNF-α (49, 50) by a mechanism regulated by the VHL gene (27). Indeed, VHL is mutated in >50% of clear cell renal cell carcinomas, and the loss of the VHL protein is associated with elevated TNF-α mRNA expression (27).

Although our studies demonstrating the apoptogenicity of TNF-induced GM2 were initially performed using the SK-RC-45 RCC cell line, it later became apparent that TNF-α had an equivalent effect on cancer lines derived from other RCC tumors. The SK-RC-54 line neither expressed abundant levels of GM2 nor was apoptogenic in the absence of TNF-α, but like SK-RC-45, acquired both characteristics when stimulated with the cytokine. Interestingly, SK-RC-26b constitutively synthesized GM2 and killed cocultured resting T cells without the need for exogenous TNF-α stimulation, but both traits could be abrogated if the tumor cells were pretreated with siRNAs to TNF-α. Indeed, SK-RC-26b was determined by RT-PCR analysis to be constitutively elaborating TNF-α, thus explaining the constitutive GM2 production and apoptogenic phenotype of the line.

Malignant transformation is associated with elevated levels of gangliosides, including GM2, GD2, and GD3 (11, 51). GM2, for example, is overexpressed in gastrointestinal tumors (52), lung (53), melanomas, and renal cell carcinomas (13). In this study, the significant TNF-α-induced increase in GM2 levels was accompanied by changes in the expression of other gangliosides too, as follows: one eluting off the HPLC column between GM2 and GM1, and another eluting after GD3 were elevated. ELISA studies also identified GD1a as a ganglioside whose accumulation was augmented by TNF. TNF-α treatment of SK-RC-45 additionally caused a reduction in GM3 expression, as well as a loss of GM1. Similar results were observed when the SK-RC-54 line was incubated with rTNF-α for 5 days (data not shown). Whether the other gangliosides induced by TNF-α are also apoptogenic for T cells is under investigation.

The increased expression of GM2 on several tumor types has made this molecule, as well as other overexpressed gangliosides, potential targets for antitumor therapy. Indeed, mAbs with specificity for GM2 bind epitopes on a large number of tumor lines, but do not recognize normal primary lines or most normal tissues (24). These Abs have been shown to mediate both complement-dependent cytotoxicity and Ab-dependent cellular cytotoxicity (53), which variously block the proliferation of some tumor cells in vitro, inhibit the outgrowth of micrometastases (54), and/or prevent the establishment of murine tumors in vivo (24, 55, 56). Mixed results, however, have been reported for related human trials, as follows: whereas anti-GM2 mAbs have been found to successfully eradicate melanoma metastases (57), in some cases anti-murine Ab responses are induced that interfere with and inhibit efficacy of the treatment (58).

Collectively, our present findings suggest that TNF-α contributes to the inhibition of T cell survival in renal cancer tissue by inducing apoptogenic gangliosides such as GM2, providing tumors a means for inhibiting the development of effective anti-tumor immune responses. Other studies to be reported separately (C. Hilston, K. Biswas, J. Finke, and C. Tannenbaum,
manuscript in preparation) demonstrate an additional mechanism by which TNF appears to synergize with tumor-derived gangliosides to induce T cell apoptosis, as follows: by suppressing TNF-induced NF-κB activation in T cells, tumor gangliosides appear to render the lymphocytes susceptible to the receptor-dependent apoptotic pathway activated by that cytokine. In fact, the two mechanisms may be operational, with TNF both enhancing tumor ganglioside synthesis and triggering the apoptosis of the consequently, ganglioside-sensitized lymphocytes. Together, these investigations suggest that antitumor vaccines might be more efficacious if administered in conjunction with therapies that neutralize gangliosides, which, by ablating tumor-induced T cell death, could possibly enhance antitumor immunity.

Disclosures

The authors have no financial conflict of interest.

References


