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Elevated Levels of Select Gangliosides in T Cells from Renal Cell Carcinoma Patients Is Associated with T Cell Dysfunction

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Increased expression of gangliosides by different tumor types including renal cell carcinoma (RCC) is thought to contribute to the immune suppression observed in cancer patients. In this study, we report an increase in apoptotic T cells from RCC patients compared with T cells from normal donors that coincided with the detection of T cells staining positive for GM2 and that the apoptosis was predominantly observed in the GM2+ but not the GM2− T cell population. Ganglioside shedding from tumor rather than endogenous production accounts for GM2+ T cells since there was no detectable level of mRNA for GM2 synthase in RCC patient T cells and in T cells from normal healthy donors after incubation with either purified GM2 or supernatant from RCC cell lines despite their staining positive for GM2. Moreover, reactive oxygen species as well as activated caspase 3, 8, and 9 were predominantly elevated in GM2+ but not GM2− T cells. Similarly, increased staining for GD2 and GD3 but not GD1a was detected with patient T cells with elevated levels of apoptosis in the GD2+ and GD3+ cells. These findings suggest that GM2, GD2, and GD3 play a significant role in immune dysfunction observed in RCC patient T cells.

Immune responses are initiated to tumor-associated Ags in cancer patients; however, these responses are relatively ineffective as evident from the continued tumor growth and disease progression (1, 2). Tumors have utilized various mechanisms to evade the immune system (1, 3). For example, tumor cells can promote immune escape by causing destruction of immune T effector cells (4–7). Indeed, a significant percentage of tumor-infiltrating lymphocytes (TILs)3 were found to be apoptotic in some tumor types, including renal cell carcinoma (RCC).

Tumor cells likely promote apoptosis of T cells in vivo since T lymphocytes undergo the same physiologic changes associated with apoptosis following in vitro culture with RCC cell lines (5, 6, 8). Various mechanisms are proposed by which tumors can induce T cell apoptosis. Tumors express elevated levels of TNF-related ligands (i.e., Fas ligand, TRAIL, and CD70), which can induce T cell apoptosis in a receptor-dependent manner (4, 5, 9). Expression of immunosuppressive costimulatory molecules like B7-H1 can induce T cell apoptosis (10) or suppress IFN-γ (Th1) response in T cells (10). T cells can also be suppressed in tumor-bearing hosts by CD4+CD25high Foxp3+ regulatory T cells (11). As a result of tumor-induced changes in myelopoiesis, a heterogeneous population of myeloid cells with suppressive activity are elevated in cancer patients (12). These myeloid cells are reported to inhibit T cell function directly as well as indirectly via the induction of regulatory Treg cell formation (13, 14).

Overexpression of select gangliosides in different tumor types has been reported (6, 15–17). In addition to promoting tumor growth and metastasis (18), gangliosides produced by tumor cells including GM2 may enhance tumor growth indirectly by suppressing immune cell function. Several reports have suggested inhibition of multiple steps in cellular immune responses by gangliosides, including Ag processing and presentation, T cell proliferation, and production of cytokines, such as IFN-γ and IL-4 (6, 19–22). Additional studies have shown that gangliosides derived from either RCC lines as well as from RCC tumor explants can either sensitize T cells to activation-induced cell death (23) or induce T cell apoptosis directly (6, 24). Although it is recognized that some ganglioside can inhibit in vitro dendritic cell function and the production of a type 1 (IFN-γ) T cell response, it is also clear that some gangliosides can stimulate an immune response. Indeed, endogenous humoral immune responses to different gangliosides have been demonstrated in some cancer patients. Gangliosides such as GM2 have been targets for immunotherapy using mAbs or vaccine to stimulate anti-GM2 Abs (25, 26). Clinical trials demonstrating induction of anti-GM2 Abs were associated with better prognosis; however, the overall response rate was low (27). Thus, under the appropriate conditions select gangliosides can be both immune stimulatory and immunosuppressive.

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3 Abbreviations used in this paper: TIL, tumor-infiltrating lymphocyte; RCC, renal cell carcinoma; FLICA, fluorochrome inhibitors of caspase; 7-AAD, 7-aminoactinomycin D; NKL, normal kidney epithelial; ROS, reactive oxygen species.

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The findings presented here suggest that select gangliosides such as GM2, GD2, and GD3 possibly shed from tumors can bind to T cells and promote immune dysfunction. When compared with T cells from normal healthy donors, a portion of T cells from RCC patients stained positive for GM2 without expressing detectable levels of the mRNA for the enzyme GM2 synthase. We also report that T cells from RCC patients display a greater level of apoptosis without cell culture than did T cells from healthy donors and that the majority of apoptosis was observed in the GM2+ (GD2⁺ or GD3⁺) T cell populations. These in vivo findings in RCC patients could be mimicked by culturing T cells from healthy donors with supernatant from RCC cell lines that contain shed gangliosides. We propose that the shedding of some ganglioside species in cancer patients may bind to and alter T cell viability.

Materials and Methods

Reagents

A hamster anti-GM2 Ab (DMF10.167.4) was a gift from Corixa and Dr. K. Rock (University of Massachusetts Medical School, Worcester, MA) (27). Mouse anti-human GM2 and GD3 Abs were purchased from BD Pharmingen. Mouse anti-human GD1a Ab was purchased from Seikagaku Kogyo. Mouse anti-human GD2 and GD3 Abs were purchased from BD Pharmingen. Mouse anti-human GM2 Ab was obtained from Seikagaku (28). FITC-conjugated rabbit anti-mouse IgM was bought from Zymed Pharmingen. Mouse anti-human GD1a Ab was purchased from Seikagaku Kogyo. Mouse anti-human GM2 and GD3 Abs were purchased from BD Pharmingen. Mouse anti-human GM1a Ab was purchased from Seikagaku (28). FITC-conjugated rabbit anti-mouse IgM and Alexa Fluor 594 rabbit anti-mouse IgG and Alexa Fluor 488 goat anti-hamster IgG were obtained from Invitrogen. Bovine brain-deived GM2 were purchased from Matreya. Normal anti-armenian ham IgG and normal anti-mouse IgM were purchased from Santa Cruz Bio-technology and BD Pharmingen, respectively. Vectashield mounting medium containing 4′,6-diamidino-2-phenylinidole (DAP) was purchased from Vector Laboratories. Annexin Vª, 7-aminocyclooctin D (7-AAD), and CD3-FITC were obtained from BD Biosciences. HPLC grade methanol and analytical grade chloroform, isopropanol, disopropyl ether, and n-hexanol were obtained from Fisher Scientific. DioC6 was purchased from Molecular Probes. Complete medium (RPMI 1640; Cleveland Clinic Media Core) consists of 10% FBS, 2 mM L-glutamine, and 50 nM nonessential amino acid solution (Invitrogen).

Cell lines

RCC cell line SK-RC-26B was obtained from Dr. N. Bander (New York Hospital, Cornell University Medical College, New York, NY). The RCC cell line 0827LM was generated at the Cleveland Clinic from a metastatic RCC (SK-RC26B or 0827LM). In other experiments, puri- fied CD3⁺ T cells from normal donors and RCC patients were immunostained to assess expression levels of GM2, GD2, GD3, and GD1a. In both cases, 1 × 10⁶ cells were adhered onto poly-1-lysine-coated slides (Eli- tron Microscopy Science) at 37°C for 1 h. After washing with 1× PBS, fixing with 3.7% paraformaldehyde, and blocking with 1% BSA, T cells were incubated at room temperature (25°C) for 2 h with respective anti-ganglioside Abs (primary Ab). Next, cells were incubated with the appropriately labeled secondary Abs. After 3 washes with 1× PBS, the cells were mounted with Vectashield mounting medium containing 4′,6-diamidino-2-phenylinidole to visualize the nuclei. A total of 200 cells was counted to assess the percentage of T cells staining positive for a given ganglioside.

Immunocytometric analysis of PBLs and TILs for GM2 expression and apoptosis

PBMCs from normal healthy donors and RCC patients (localized and metastatic) as well as TILs were isolated by methods described earlier. T cells were then analyzed for ganglioside positivity (GM2, GD2, GD3, and GD1a) and levels of apoptosis. One × 10⁶ cells were stained with the hamster anti-human GM2 Ab (DMF10.167.4) for 30 min at 4°C. In a smaller subset of patients, peripheral blood T cells were stained with mouse anti-human GD2, GD3, and GD1a Abs (plus appropriate secondary Abs). T cells were then washed twice with FACS buffer and stained with PE-conjugated CD3 for an additional 30 min at 4°C. T cells were then washed twice with FACS buffer, resuspended in 1× PBS and stained with Annexin Vª and 7-AAD for 15 min at room temperature under constant shaking. Ten thousand events were acquired on a FACS-Calibur multivariable flow cytometer and analyzed using CellQuest version 3.3.0 software (BD Biosciences).

mRNA analysis of GM2 synthase by RT-PCR in T cells staining positive for GM2

Total RNA was isolated from RCC line SK-RC26B and T cells using the RNeasy minikit (Qiagen) according to the manufacturer’s protocol. For RT-PCR analysis of GM2 synthase and GAPDH mRNA, the Superscript First-Strand Synthesis System (Ambion) was used to synthesize the respective cDNAs. PCR was conducted using 5′-CAT CGG ATC TTC TGC CCG TTC CTA AGA GGC TTA-3′ as the sense primer and 5′-CGA GCC GCC GCG ACA GCC AGT AGA GTC TCC ACA-3′ as the antisense primer, which gave 1734-bp fragment of GM2-synthase. GAPDH primers were as follows: 5′-ACCTGGCAAGGTCATCCAT-5′ (sense) and 5′-TCCACCACCCGGTTGCTGTA-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 45 s. Electrophoresis of the samples was done on a 1.5% agarose gel and visualized by ethidium bromide staining.

Analysis of reactive oxygen species (ROS) and caspase activity in T cells

T cells were incubated in 6-well plates at 1 × 10⁶ cells/ml in the presence or absence of SK-RC26B gangliosides for 18, 48, or 72 h at 25 μg/ml. Annexin Vcentrifugated of cells was also treated with 2 μM H₂O₂ for 18 h, which was used as a positive control (data not shown). At the desired time points, cells were harvested and stained for active caspase 3, 8, and 9, ROS, annexin V7-AAD, and GM2. To study activation of caspases, cells were stained with fluorochrome inhibitors of caspases (FLICA) from Immunochemistry Technologies for 1 h at 37°C protected from light, followed by staining with hamster anti-human GM2 Ab. Induction of ROS was deter- mined by staining the cells with CM-H₂DCFDA dye from Invitrogen for 4 h at 37°C followed by staining for GM2. Apoptosis induction in SK-RC26B ganglioside-treated T cells was studied by surface staining with
hamster anti-human GM2 Ab (or anti-GD2 or anti-GD3 Abs) followed by staining with Annexin V PE and 7-AAD.

ELISA to test the specificity of different anti-ganglioside Abs

ELISA was performed to demonstrate the specificity of the hamster anti-human (DMF10.167.4) and mouse anti-human GM2 Ab (KM696) described earlier (28). Bovine brain-derived gangliosides coated on a 96-well format flat-bottom ELISA plate were immunostained with 1/H9262g/ml of the mouse anti-human GM2 Ab (KM696) followed by staining with HRP-conjugated rabbit anti-mouse IgM (secondary Ab).

Ganglioside isolation from the SK-RC-26B cell line

Gangliosides were isolated from tumor cells as described before (6), with minor modifications. Extraction of gangliosides was performed with chloroform:methanol (1:1) for 18 h at 4°C followed by partitioning in 10 ml of diisopropyl ether/1% butanol/0.1% aqueous NaCl. The lyophilized, final aqueous phase was passed through a Sephadex G-25 column to remove the salts and small molecular mass impurities. The ganglioside profile was determined by HPLC (Waters) with online electrospray ionization tandem mass spectrometry (liquid chromatography/electrospray ionization/mass spectrometry/mass spectrometry) as previously described (6).

Statistical analysis

A value of \( p \) was calculated with Student’s \( t \) test from the SEM for unpaired samples using Microsoft Excel, version 2003 software. All of the experiments were done at least three times.

Results

The ganglioside GM2 that is expressed by many RCC is also detected on a portion of T cells from RCC patients but not those from normal healthy donors

To quantitate the percentage of T cells positive for GM2 staining, PBMCs from normal donors and PBMCs from RCC patients along with TILs were incubated with anti-GM2 and anti-CD3 Abs followed by analysis using flow cytometry (Fig. 1A). A significant percentage of T cells from the peripheral blood of RCC patients but not normal donors stained positive for GM2 (mean, 15 ± 3.51%; Fig. 1A). Interestingly, TILs displayed the highest percentage of GM2-positive T cells (mean, 45.9 ± 4.9%). Fig. 1B depicts a representative density plot analysis of T cells from normal donor blood, patient blood vs TILs. Similar flow cytometry results were obtained when patient T cells and those from normal donors were stained with a second mouse anti-human GM2 Ab (KM696) that is also specific for GM2. Likewise, comparable results were observed when patient and normal donor T cells were stained with anti-GM2 Ab and examined using a fluorescent microscope. The intensity of GM2 staining per cell was measured from images captured with a Leica DMR fluorescent microscope followed by quantitation using NIH Image J software (version 1.41), which showed that T cells from RCC patients displayed a significant high intensity of GM2\(^+\) specific fluorescence when compared with that of T cells from normal volunteers (data not shown).

The GM2 detected on RCC patient T cells is not attributable to endogenous production but rather binding of exogenous ganglioside

To address the question of whether patient T cells synthesize and express GM2, total RNA was isolated from the RCC cell line SK-RC26B as a positive control and from three metastatic RCC patient T cells using an RNasey minikit (Qiagen). Electrophoresis of the cDNA following RT-PCR demonstrates no detectable message for GM2 synthase mRNA in any of the three metastatic samples. However, mRNA from SK-RC26B yielded a positive band
with the appropriate bp PCR product, indicating detectable GM2 synthase message (Fig. 2A). These findings suggest that patient T cells lack detectable expression of the message required for synthesizing the GM2 synthase enzyme. In the same experiments where mRNA was isolated, immunofluorescent staining with mouse anti-human GM2 Ab (KM696) demonstrated T cells from all three metastatic patient cells stained positive for GM2, although to varying degrees, as did the SK-RC-26B cell line (Fig. 2B). The inability to detect GM2 synthase mRNA in patient T cells does not appear to be attributable to the insensitivity of the RT-PCR assay. This was addressed by determining the number of GM2 \( \text{SK-RC-26B} \) cells (5.6, 2.8, 1.4, 0.7, and 0.35 \( \times 10^5 \) cells) that is required to detect GM2 synthase when these tumor cells are mixed with NKE cells (2 \( \times 10^6 \)) which do not express GM2 or GM2 synthase (Fig. 2C). As seen in Fig. 2C, GM2 synthase mRNA could be detected even when the GM2 \( \text{SK-RC-26B} \) line represented only 3% (0.7 \( \times 10^5 \)) of the total cell population. However, when RNA was isolated from patient T cells (5 \( \times 10^6 \)), no GM2 synthase mRNA was detected even though 38 and 24% of T cells from two patients, respectively, stained positive using anti-GM2 Ab (Fig. 2B).

To test whether exogenous GM2, possibly from tumor cells is binding to T lymphocytes, CD3/CD28-activated T cells were cocultured with purified bovine brain-derived GM2 (80 \( \mu \text{g/ml} \)) for varying time points (24–96 h) in the presence of IL-2 as shown in FIGURE 2.

FIGURE 2. GM2 synthase message is absent in RCC metastatic patient T cells, although they stain positive for GM2. mRNA was isolated from metastatic patient T cells and also from the cell line SK-RC26B by methods described earlier. RT-PCR was done to detect the levels of GM2 synthase mRNA. GAPDH was used as the housekeeping gene (A). Both SK-RC-26B cells and RCC patient T cells were subsequently stained with mouse anti-human GM2 Ab to detect the presence of GM2 by fluorescent microscopy (B). NKE cells were mixed with varying percentage of the RCC cell line SK-RC-26B. The percentage of GM2 \( \text{SK-RC-26B} \) cells (as calculated from flow cytometry analysis; data not shown) is shown in C. mRNA was isolated from the NKE/SK-RC-26B cell mixture using a Qiagen RNeasy kit as described earlier. RT PCR was done to detect levels of GM2 synthase mRNA. \( \beta\text{-Actin} \) was used as the endogenous control. Data demonstrate that GM2 synthase message can be detected from a population containing as low as 3% GM2-positive cells (C).

FIGURE 3. Peripheral blood T cells from healthy donors do not express the message for GM2 synthase, although they stain positively for GM2 when incubated with either purified bovine brain-derived GM2 or with supernatants derived from RCC cell lines. CD3/CD28-activated T cells were cultured with 100 \( \mu \text{g} \) of purified bovine brain-derived GM2 for 24–96 h. Cells were stained with mouse anti-human GM2 (KM696) Ab for immunofluorescent detection of GM2 by microscopy (A), and mRNA was isolated from these T cells to assess GM2 synthase mRNA by RT-PCR (B). Results indicate detectable GM2 staining by microscopy (A), although no GM2 synthase message was found to be present in the T cells treated with GM2 (B). Similarly, T cells that were cultured with conditioned medium from RCC cell lines SK-RC-26B and 0827LM show significant GM2 staining (as indicated by green fluorescence) by microscopy (C); no detectable GM2 synthase was found in RNA isolated from the tumor-conditioned medium-treated T cells (D).
Fig. 3. T cells were then immunostained with mouse anti-human GM2 Abs (KM696 and DMF10.163.4) to detect GM2. SK-RC26B cells were also immunostained for GM2 in parallel as a positive control (Fig. 3A). A time-dependent increase in GM2 positivity of the T cells after incubation with purified bovine brain-derived GM2 (Fig. 3A) was observed. RT-PCR analysis of the RNA isolated from these T cells shows no detectable message for GM2 synthase in comparison to that from the SK-RC26B line (Fig. 3B). We also tested whether T cells from RCC patients and healthy donors when incubated with purified GM2 would have a similar ability to bind GM2. Using T cells isolated from two RCC patients and two healthy donors, we did not observe an appreciable difference in the percentage of GM2 staining cells (data not shown).

Additionally, immunofluorescent staining using the mouse anti-human GM2 Ab (KM696) demonstrates significant levels of GM2-positive T cells (Fig. 3C) after culture with supernatants from either 0827LM (24%) or SK-RC26B (33%) cell lines when compared with medium control T cells (0%), indicating transfer of GM2 from RCC cell line-derived supernatants. Data from RT-PCR analysis show no detectable message for GM2 synthase in T cells treated with or without RCC supernatants (Fig. 3D), confirming that GM2 detected on the T cells is transferred from conditioned RCC supernatants during coculture.

Gangliosides shed from the SK-RC-26B cell line induce ROS and activate caspases and apoptosis predominantly in the GM2\(^{+}\) T cell population. T cells were incubated with 15 \(\mu\)g/ml SK-RC-26B gangliosides for 18, 48, and 72 h and stained with H\(_2\)DCFDA (A, left panel), a dye that stains for ROS induction and FLCIA 3, 8, and 9 (A, right panel). Results indicate induction of ROS in T cells treated with SK-RC-26B gangliosides, as indicated by the increased mean fluorescence intensity of H\(_2\)DCFDA, which doubled in 18 h over background and then increased thereafter until 72 h (A, top left panel). Caspases 3, 8, and 9 were also increased at 18 h, which reached maximum activity at 48 h (B, top right panel). Double staining of the T cells treated with SK-RC-26B gangliosides with anti-GM2 Ab and FLCIA demonstrate that caspase activity was predominantly present in GM2\(^{+}\) T cells over GM2\(^{-}\) T cells (B, bottom left panel). Similar results were observed when T cells were assessed for apoptosis following triple staining of the cells with anti-GM2 Ab, annexin V, and 7-AAD, showing increased apoptosis mainly in the GM2\(^{+}\) T cell population rather than the GM2\(^{-}\) T cells (B, bottom right panel).
and 9 that was detectable at 18 h and reached maximum activity at 48 h (top right panel; Fig. 4A). By double staining T cells with anti-GM2 Ab plus FLICA to detect active caspases, we noted that the majority of the caspase activity was present in GM2⁺ T cells rather than the GM2⁻ T cells (Fig. 4B, bottom left panel). Similar results were observed when T cells were assessed for apoptosis following staining with annexin V, 7-AAD, and GM2 Ab. When T cells were cultured with SK-RC-26B gangliosides, apoptosis increased to 49% compared with T cells cultured in medium alone (17%). Analysis of annexin V/7-AAD staining in both the GM2⁺ and GM2⁻ T cell populations demonstrated significantly more GM2⁺ apoptotic T cells as indicated by annexin V⁺/7-AAD⁺ T cells compared with the GM2⁻ counterpart (C).

 Representatives density plots showing apoptosis in GM2⁺ and GM2⁻ T cells are shown in D.

 FIGURE 5. GM2⁺ T cells from RCC patients display a heightened level of apoptosis. PBMCs isolated from RCC patients and normal donors along with TILs were immunostained for CD3, GM2, annexin V, and 7-AAD. A shows that peripheral blood T cells and TILs demonstrate a heightened level of apoptosis, as indicated by the annexin V⁺/7-AAD⁺ T cell population when compared with normal donor T cells. B represents density plots from a single experiment illustrating increased levels of apoptosis in T cells derived from TILs and patient peripheral blood. Analysis of the GM2⁺ vs GM2⁻ T cell populations demonstrated significantly more GM2⁺ apoptotic T cells as indicated by annexin V⁺/7-AAD⁺ T cells compared with the GM2⁻ counterpart (C). Representative density plots showing apoptosis in GM2⁺ and GM2⁻ T cells are shown in D.

To determine whether exogenous GM2 bound to patient T cells in vivo is associated with T cell death, PBMCs isolated from the peripheral blood of RCC patients and normal donors along with TILs were immunostained for CD3, GM2, annexin V, and 7-AAD. Flow cytometric analysis revealed that peripheral blood T cells from RCC patients demonstrated a higher level of apoptosis (mean, 21 ± 3.02%) when compared with that of normal donor T cells (mean, 5 ± 0.83%; Fig. 5A). Interestingly, TILs demonstrate an even more heightened level of apoptosis as indicated by the percentage of CD3⁺ annexin V⁺/7-AAD⁺ lymphocytes (mean, 53 ± 4.9%; Fig. 5A). Density plots from representative experiments illustrate the increased level of apoptosis in T cells derived from TILs and patients’ peripheral blood (Fig. 5B). We also noted
that the percentage of apoptotic T cells in the peripheral blood of patients was significantly higher in the GM2\(^+\) (mean, 61 ± 5.9%) than the GM2\(^-\) (mean, 16 ± 2.4%) population (Fig. 5C). A similar trend was observed for TILs. A significantly higher percentage of GM2\(^+\) TILs (mean, 77 ± 4.6%) were apoptotic compared with GM2\(^-\) TILs (mean, 36 ± 6.3%; Fig. 5C). However, a significant level of apoptosis was found in the GM2\(^-\) TILs suggesting that other tumor-derived cells or products play a role in T cell death within the tumor microenvironment. Representative density plots showing apoptosis of GM2\(^+\) and GM2\(^-\) T cells (from peripheral blood and tumor) are shown in Fig. 5D.

Other gangliosides beside GM2 promote T cell apoptosis in RCC patients

We determined whether other gangliosides might also be increased in expression on RCC patient T cells compared with T cells isolated from healthy donors. As seen in Fig. 6A, ~50% of T cells from normal donors stained positive for GD1a and the percentage of GD1a\(^+\) T cells was not significantly different when compared with the staining of RCC patient T cells. However, RCC patient T cells did show a dramatic increase in the levels of both GD2\(^+\) and GD3\(^+\) T cells (n = 14; Fig. 6A) relative to normal donor T cells (n = 16). Very similar data were obtained with immunofluorescence staining of T cells attached to poly-L-lysine slides and counting 200 cells to assess the percentage of ganglioside-positive cells (Fig. 6B).

To assess whether GD2 and GD3 positivity in T cells of RCC patients is correlated with apoptosis, four-color staining (CD3, GD2, or GD3, annexin V, and 7-AAD) was performed (n = 11). Gating within the CD3\(^+\) population for GD2\(^+\) and GD2\(^-\) cells revealed that GD2\(^+\) T cells are significantly more apoptotic (mean, 57 ± 16.2%) than GD2\(^-\) (mean, 16 ± 3.9%) cells (Fig. 7A). Likewise, a significantly higher percentage of GD3\(^+\) T cells (mean, 58 ± 15.1%) stained positive for annexin V/7-AAD relative to the GD3\(^-\) cell population (mean, 19 ± 5.7%; Fig. 7B). These findings suggest that GM2, GD2, and GD3 but not GD1a can contribute to T cell apoptosis observed in RCC patients.

Discussion

Overexpression of various gangliosides has been reported in different histological tumor types including RCC (15–17, 29, 30), likely resulting from modifications in the expression of key enzymes regulating the rate-determining step of ganglioside biosynthesis (30, 31). Tumor-derived gangliosides have been shown to inhibit development of antitumor immune responses in vivo in several different murine models (32). Additionally, studies in vitro with bovine brain-derived GM2 demonstrated suppressed dendritic cell function (33) and, in a separate study, GM2 induced apoptosis of a cytotoxic T cell line (34). The first report suggesting the immunosuppressive role of tumor-derived GM2 in RCC patients came from our laboratory, which showed that GM2 present in RCC tissues and cell lines not only induced T cell apoptosis in vitro, but also suppressed stimulus-induced production of IFN-γ in T cells at lower concentrations (100 ng/ml) (6). In this study, we provide in vivo data indicating the immunosuppressive role of this ganglioside in tumor-bearing hosts. Our findings suggest that...
GM2, which is expressed on many RCCs, can be shed from the tumor with subsequent binding to patient T lymphocytes resulting in apoptosis.

Immunostaining of human tissue with a mAb to GM2 (KM696) (35) demonstrated that a wide variety of epithelial malignancies express this ganglioside. GM2 is also expressed on normal epithelial tissue at secretory borders as well as the brain but is not expressed on normal connective tissue or immune tissue such as spleen and lymph nodes (35). In this study, we confirm that GM2 is not detected on T cells from the peripheral blood of healthy donors. However, our findings did show that a significant percentage of peripheral blood T cells from RCC patients (mean, 15 ± 3.5%) stained positive for GM2 and the percentage was even greater when staining tumor-infiltrating T cells (mean, 46 ± 4.98%; Fig. 1). However, the GM2-positive T cells present in the peripheral blood of RCC patients did not appear to synthesize their own GM2 since RT-PCR analysis failed to detect message for GM2 synthase, the key enzyme that regulates GM2 synthesis, even though mRNA for this enzyme was readily detected in the SK-RC-26B cell line (Fig. 2A).

The presence of GM2+ T cells from RCC patients in the absence of any detectable levels of mRNA for GM2 synthase leaves open the possibility that GM2 was shed from patient tumors and then taken up by T cells as visualized by immunostaining. This hypothesis is supported by in vitro studies showing that when T cells were incubated with either purified bovine brain-derived GM2 (Fig. 3A) or with conditioned supernatants from SK-RC-26B or 0827LM cell lines (Fig. 3C), a significant number of T cells that were initially negative for GM2 stained positive for GM2 starting at 24 h after initiation of cultures. Moreover, in both the experiments, the message for GM2 synthase was absent from the lymphocytes, thereby confirming the notion that GM2 detected on the T cells is likely shed from RCC tumors (Fig. 3, B and D). As shown in Fig. 3A, the vast majority of the SK-RC-26B cells (and 0827LM; data not shown) stain positive for GM2 and additional studies have shown that the supernatants obtained from 4-day cultures of the RCC lines (SK-RC-26B and 0827LM) contained GM2 as detected by HPLC-mass spectrometry (data not shown).

Several groups have earlier reported significantly higher levels of gangliosides in the plasma and serum of patients with different cancers (16, 36). Therefore, it is possible that GM2 is elevated in the serum of RCC patients which could then bind to T cells. We are currently testing whether serum from RCC patients can transfer GM2 to peripheral blood T cells. We have recently found that exogenous, purified bovine brain-derived GD3 and RCC-derived gangliosides are most readily internalized in activated T cells when compared with naive resting T lymphocytes (37). These findings may explain why not all of the T cells stain positive for GM2 in either the peripheral blood or tumor of RCC patients.

Previous work from our laboratory showed that GM2-expressing RCC cell lines along with tumor supernatants induced apoptosis in peripheral blood T cells from healthy donors and that addition of neutralizing Ab partially blocked apoptosis by >50% (6). In this study, we show that incubating T cells from normal donors with purified RCC cell-derived gangliosides can directly induce ROS formation, caspase activation, and the induction of apoptosis. As seen in Fig. 4, the majority of the apoptosis was confined to the GM2+ stained T cells and not the GM2− population. Data presented here also show that without cell culturing, patient T cells freshly isolated from the blood or tumor demonstrated a higher level of apoptosis relative to T cells from healthy donors. Our finding in RCC patients is consistent with those of others that reported increased apoptotic activity of T cells from patients with squamous cell carcinoma of the head and neck as well as gliomas (38). Furthermore, analysis of our four-color flow cytometry data revealed that a significantly higher percentage of the GM2+ T cells were apoptotic compared with the GM2− population (Fig. 5B), thereby implying GM2’s potential role in T cell apoptosis observed in RCC patients.

Our studies also identified two additional gangliosides that may be possible mediators of T cell apoptosis observed in RCC patients. Both immunofluorescent staining and flow analysis showed that a higher percentage of RCC patient T cells stain positive for GD3 and GD2 when compared with T cells from normal donors (Fig. 6). Moreover GD3 is reported to induce apoptosis in various cell types (39). Interestingly enhanced expression of GD3 has been observed in different tumors in both human and animal models (40). Likewise, GD2 is expressed by different tumor types and has been implicated in immune dysfunction (33). Four-color analysis of our flow cytometry data demonstrates a much higher percentage of apoptotic T cells in the CD3+GD2+ as well as CD3+GD3− populations relative to that of CD3+GD2− and CD3+GD3+ cells (Fig. 7), clearly indicating that GD2 and GD3 expression on patient T cells is associated with T cell death. Overall, our findings suggest that binding of tumor-derived gangliosides to T cells resulting in apoptosis represents a means by which tumor cells may escape the immune system. Thus, targeting gangliosides for immune therapy in an attempt to reverse immune suppression in cancer, combined with dendritic cell-based vaccines pulsed with tumor-derived peptides may be a viable therapeutic approach.

Disclosures
The authors have no financial conflict of interest.