GM2 Expression in Renal Cell Carcinoma: Potential Role in Tumor-Induced T-Cell Dysfunction

Kaushik Biswas,1 Amy Richmond,1 Patricia Rayman,1 Soumika Biswas,1 Mark Thornton,1 Gaurisankar Sa,1 Tanya Das,1 Renliang Zhang,2 Ali Chahlavi,3,4 Charles S. Tannenbaum,1 Andrew Novick,4 Ronald Bukowski,1 and James H. Finke1,3

1Department of Immunology and Mass Spectrometry Core, Lerner Research Institute; 2Department of Neurosurgery; 3Glickman Urological Institute; Experimental Therapeutics, Cleveland Clinic Foundation, Cleveland, Ohio; and 4Bose Institute, Calcutta, India

Abstract

Multiple mechanisms have been proposed to account for immune escape by tumors. Although gangliosides have long been known to suppress T-cell immunity, few studies have examined the effect of human tumor-derived gangliosides on immune responses. Here, we show that gangliosides isolated from renal cell carcinoma (RCC) cell lines and clear cell tumor tissue can induce apoptosis in peripheral blood T cells. The RCC tissue-derived gangliosides also suppressed IFN-γ and, in many cases, interleukin-4 production by CD4+ T cells at concentrations (1 ng/mL–100 pg/mL) well below those that induce any detectable T-cell death (4–20 μg/mL). Additional findings show that GM2 expressed by RCC plays a significant role in promoting T-cell dysfunction. This is supported by the demonstration that all RCC cell lines examined (n = 5) expressed GM2 as did the majority of tumors (15 of 18) derived from patients with clear cell RCC. Furthermore, an antibody specific for GM2 (DMF10.167.4) partially blocked (50–60%) T-cell apoptosis induced by coculturing lymphocytes with RCC cell lines or with RCC tissue-derived gangliosides. DMF10.167.4 also partially blocked the suppression of IFN-γ production induced by RCC tissue-derived gangliosides, suggesting that GM2 plays a role in down-regulating cytokine production by CD4+ T cells. (Cancer Res 2006; 66(13): 6816-25)

Introduction

Although immune responses to tumor antigens are initiated in cancer patients, it is evident that these responses are generally ineffective as evident by continued tumor growth and disease progression (1, 2). It is also known that tumor cells can promote immune evasion by causing the destruction of immune effector cells (3–6). Indeed, a significant percentage of tumor-infiltrating T lymphocytes were found to be apoptotic in some tumor types, including renal cell carcinoma (RCC; ref. 4). Additional data from our laboratory and others have now confirmed that the tumor cells themselves that mediate these effects because, when cocultured in vitro, cancer cell lines can be clearly shown to induce apoptosis of T lymphocytes (4, 5).

Several reports focusing on the mechanisms by which the tumor cells induce T-cell apoptosis suggest that histologically disparate tumors express elevated levels of various tumor necrosis factor (TNF)-related ligands (i.e., Fasl, TNF-related apoptosis-inducing ligand, and CD70), which can induce apoptosis of T cells in a receptor-dependent fashion (3, 4, 6–8). It also seems that the expression of the immunosuppressive costimulatory molecule B7-H1 by some tumors may induce apoptosis in activated T cells expressing the receptor PD-1 (9). Additional studies from our laboratory have shown that gangliosides expressed by RCC lines, as well as those isolated from the supernatants of renal tumor explants, can either sensitize T cells to activation-induced cell death or directly induce T-cell apoptosis via a mechanism that involves suppression of nuclear factor-κB (NF-κB) activation (10, 11).

Gangliosides are structurally diverse acidic glycosphingolipids present in the outer leaflet of the plasma membrane of cells, which are composed of sialic acid, carbohydrates, and ceramide (12). Gangliosides exist as clusters on the cell surface and contribute to the structure of microdomains referred to as lipid rafts (13) and function as signaling intermediates in the regulation of multiple cellular responses by modulating the activities of various receptors (14, 15). Enhanced production of gangliosides has been observed in different tumor types, and in many cases, the changes in individual ganglioside expression have been identified (16–18). For example, malignant melanoma and neuroblastoma cells overexpress GD3, GD2, and GM2, which are actively shed into the tumor microenvironment (16, 18). Gangliosides have been implicated in tumor progression by promoting tumor-associated angiogenesis (19) and regulating cell adhesion/motility, thereby initiating tumor metastasis (14, 15). GM2/GD2 synthase mRNA expression is increased in human gastrointestinal cancers, suggesting a role for GM2 in this disease (20). GM2 expression has been directly related to the tumorigenicity (17) and metastatic potential of human melanoma. Recently, GM2 synthase expression and, consequently, GM2 have been suggested to play a major role in the motility and invasiveness of RCC (21). Gangliosides have also been found to inhibit multiple steps in the cellular immune responses, including antigen processing and presentation, T-cell proliferation, and production of cytokines, such as IFN-γ (22–26). In spite of the reports relating GM2 to tumor progression and metastasis (17, 21), there are only a few studies implicating GM2 in tumor-induced immune suppression. Data presented in this article suggest that GM2 is one of the major gangliosides expressed by RCC and that it may play an important role in tumor-mediated immune dysfunction in RCC patients.

Requests for reprints: James H. Finke, Department of Immunology, NB30, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH 44195, Phone: 216-444-5186; Fax: 216-444-9329; E-mail: finkej@ccf.org.

1Department of Immunology and Mass Spectrometry Core, Lerner Research Institute; Cleveland Clinic Foundation; Cleveland, Ohio; and 2Bose Institute, Calcutta, India.

doi:10.1158/0008-5472.CAN-06-0250

© 2006 American Association for Cancer Research.

Cancer Res 2006; 66: (13). July 1, 2006 6816 www.aacrjournals.org

Downloaded from cancerres.aacrjournals.org on April 20, 2017. © 2006 American Association for Cancer Research.
Materials and Methods

Reagents. A hamster monoclonal anti-GM2 antibody (DMF10.167.4) was a gift from Corixa Corp. (Seattle, WA) and Dr. Kenneth Rock (Department of Pathology, University of Massachusetts Medical School, Worcester, MA; ref. 27). Ficoll-Hypaque was purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Anti-human CD41 tetramer and human T-cell enrichment cocktail were obtained from StemCell Technologies (Vancouver, British Columbia, Canada). Standard ganglioside mixtures and GM2 were purchased from Matreya (Pleasant Gap, PA). Peroxidase-conjugated affinity-purified anti-hamster IgG was purchased from Rockland (Gilbertsville, PA). Biotin-conjugated rabbit anti-mouse IgG and streptavidin-conjugated peroxidase were obtained from Jackson Immunoresearch (West Grove, PA). Polyisobutylmethacrylate was obtained from GlycoTech (Rockville, MD). 4-Chloro-1-naphthol was purchased from Calbiochem (La Jolla, CA). Annexin V was obtained from BD Biosciences (San Jose, CA). Precoated LPHPK silica gel 60 Å high-performance thin-layer chromatographic (HPTLC) plates were obtained from Whatman, Inc. (Clifton, NJ). Supelcosil LC-NH2 column for high-performance liquid chromatography (HPLC) was purchased from Sigma-Supelco (Bellefonte, PA). HPLC grade acetonitrile and methanol and high-performance liquid chromatography (HPLC) was purchased from Whatman (Clifton, NJ). nylon-3 was purchased from Molecular Probes (Eugene, OR).

Cell lines. RCC cell lines SK-RC-45 and SK-RC-26B were obtained from Dr. Neil Bander (The New York Hospital, Cornell University Medical College, New York, NY). The SK-RC-54V cell line used in this article was derived from the SK-RC-54 cell line (obtained from Dr. Bander) and differs from the parent line in that it is apoptogenic for T cells and expresses GM2. The RCC cell line 0827 Lung Met was generated at the Cleveland Clinic Foundation as a patient with RCC and was immortalized by transfection with the gene for telomerase (Dr. Katerina Gurova and Andrei V. Gudkov, Department of Molecular Genetics, Cleveland Clinic Foundation). These cells were maintained in complete RPMI 1640 at 37°C with 5% CO2 and allowed to reach confluence in 150-mm dishes before use in coculture experiments with peripheral blood T lymphocytes.

RCC Tissues. Tissue from primary lesions of RCC resected tumors was provided by the Cooperative Human Tissue Network (funded by the National Cancer Institute). Informed consent (Institutional Review Board (IRB) 1382) was obtained from all patients with localized disease. The tumor specimens included 32 patients diagnosed with clear cell RCC (stage pT1a in 17 patients and stage T2 in 15 patients). Blood specimens were obtained from healthy volunteers with informed consent (IRB 1382). Blood was centrifuged over a Ficoll-Hypaque density gradient to obtain total leukocytes, from which T cells were purified by negative magnetic selection using microbeads coated with antibodies (human T-cell enrichment cocktail) to remove macrophages, natural killer cells, B cells, and BCCs. The T-cell isolation procedure yielded cells that were >97% positive for CD3 as defined by immunocytometry.

Isolation of gangliosides. Gangliosides were isolated from RCC tissues or RCC cell lines essentially as described before (10, 28) with minor modifications. Briefly, RCC tissues were homogenized in 1× PBS (10%) and subjected to lyophilization. The lyophilized powder was then extracted with chloroform-methanol (1:1) for 18 hours at 4°C followed by partitioning in 10 mL diisopropyl ether/1-butanol (0.1% aqueous NaCl). The lyophilized, final aqueous phase was passed through a Sephadex G-25 column to get rid of the salts and small molecular weight impurities. Isolated gangliosides eluting in the void volume were lyophilized and redissolved in chloroform-methanol.

HPTLC analysis of isolated gangliosides. HPTLC analysis of isolated gangliosides was done using precoated LPHPK silica gel 60 Å HPTLC plates measuring 10 × 10 cm (10, 28). The plates were developed in chloroform/ methanol/0.25% CaCl2 (60:40:5, v/v/v). Gangliosides were visualized as purple bands by spraying with resorcinol-HCl reagent. Bovine brain-derived gangliosides (GM3, GM2, GM1, GD1a, GD1b, and GT1b) were run on lanes of the HPTLC plates as standards.

Immunostaining of TLC plates. Standard bovine brain-derived gangliosides GM3, GM2, GM1, and GD3 were separated on aluminum-backed silica gel plates, and the plates were dipped in 0.2% solution of polysorbate/N-methacyrlate in acetone for 2 minutes. Plates were then blocked with PBS-1% human serum albumin, washed with 1× PBS, and dried in a vacuum dessicator. Immunostaining was done with human anti-hamster GM2 antibody (IgG; ref. 29).

HPLC of gangliosides. HPLC of the isolated gangliosides was done using a Beckman Coulter (Fullerton, CA) HPLC and a Supelco Supelcosil LC-NH2 column (25 cm, 100 Å; ref. 30). The separation was carried out with a gradient of the following solvent mixtures used as the mobile phase: solvent A [acetonitrile, 5 mmol/L phosphate buffer, pH 5.6 (83:17)] and solvent B [acetonitrile, 20 mmol/L phosphate buffer, pH 5.6 (50:50)]. The gradient elution program was the same as described earlier (30). 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 in a Beckman Coulter System Gold 168 detector.

Immunofluorescence. The assessment of GM2 expression by RCC tissue-derived cells was done on short-term cultures. Briefly, RCC tissues were digested with collagenase for 1 hour, and single cells were allowed to adhere (24 hours) in complete RPMI 1640 containing 10% fetal bovine serum. The adherent cells were trypsinized and grown on chamber slides until 80% confluent. Thereafter, cells were washed with PBS, fixed with 3.7% paraformaldehyde, and washed with PBS followed by treatment with 0.05% Triton. The cells were then blocked with 1% bovine serum albumin in PBS followed by incubation with anti-GM2 antibody (DMF10.167.4). Cells were then incubated with FITC-conjugated secondary antibody before quick-fixing cells in methanol and staining the nuclei with 4,6-diamidino-2-phenylindole (DAPI). Microscopic analysis was done with a confocal microscope, and pictures were recorded using Image-Pro software.

Induction and analysis of peripheral blood T-cell apoptosis. T cells (1 × 106/mL) were cultured with or without gangliosides for 72 hours. To assess the apoptotic activity of RCC lines, T cells were cocultured with SK-RC cells at a tumor to T-cell ratio of 3:1. Following a 72-hour incubation period at 37°C, T cells were removed from the SK-RC monolayer by gentle washing and assessed for viability by trypan blue staining. Quantitation of DNA fragmentation in T cells was determined by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay (Apo-BrdU kit, Phoenix Flow Systems, San Diego, CA; ref. 4). In some experiments, apoptosis of T cells was determined by staining cells with Annexin V and 7-aminoactinomycin D (7-AAD; BD PharMingen, San Diego, CA). After staining, events were acquired on a multivariable FACScan, and analysis was done using quadrad software (CellQuest v3.3, Becton Dickinson, San Jose, CA).

Detection of mitochondrial permeability transition in T cells. T cells were cultured with RCC-derived gangliosides for 72 hours, following which cells were stained with a mitochondrial dye, DiOC6. Cells were then fixed, permeabilized, and subsequently stained, events were acquired on a multivariable FACScan, and analysis was done using quadrad software (CellQuest v3.3, Becton Dickinson, San Jose, CA).

Measurement of GM2 expression by RCC cell lines using flow cytometry. Cell lines (2 × 106 per tube) were stained with hamster anti-human GM2 antibody (DMF10.167.4) or IgG isotype control followed by incubation with a FITC-conjugated goat anti-hamster IgG. After washing, the cells were fixed in 1% paraformaldehyde. At least 20,000 events were then acquired using a FACScan, and analysis was done using CellQuest v3.3 software.

Measurement of Th1/Th2 response in T cells exposed to RCC gangliosides and GM2 by flow cytometry. Isolated T cells were cultured with various gangliosides for 24 hours before stimulating the cells for an additional 48 hours using either cross-linked anti-CD3 plus anti-CD28 antibodies or with phorbol 12-myristate 13-acetate (PMA; 10 ng/mL) plus ionomycin (0.75 pg/mL). Cells were then labeled with anti-CD3 antibody to determine the T-cell population or with anti-CD4 antibody to detect the CD4+ T-cell subset. Cells were then fixed, permeabilized, and subsequently stained for the CD4+ T-cell subset using an anti-CD4 antibody.
stained with antibodies to IFN-γ and interleukin (IL)-4. Cells were acquired on BD LSR, and analysis was done using FloJo software.

**Lipid-bound sialic acid assay.** The assay to measure plasma total ganglioside [expressed as lipid-bound sialic acid (LSA)] was carried out using the resorcinol reagent as reported previously (31). Briefly, gangliosides (20 μL) from RCC tissue or RCC cell lines were dissolved in 200 μL cold distilled water and the LSA content was quantitated by resorcinol-HCL reagent. The concentration of LSA was determined by the absorption at 580 nm compared with the standard curve generated using known amounts of free sialic acid (N-acetylneuraminic acid; ref. 31).

**Statistical analysis.** Student’s t test (paired two sample for mean or two sample using equal variances) was used to determine P using Microsoft Excel software (version 2003). SE was calculated from SD using Microsoft Excel software.

Results

**RCC cell lines and isolated gangliosides induce T-cell death.** Coculture experiments showed that monolayers of RCC lines can induce T-cell death after 72 hours of incubation. RCC cell lines SK-RC-26B, 0827 Lung Met, and SK-RC-54V induced anywhere between 27% and 35% T-cell death, whereas T cells cultured in medium alone or cocultured with an immortalized NKE cell line displayed only background levels of T-cell death (Fig. 1A). Because our laboratory showed previously a role for gangliosides in RCC cell line-mediated apoptosis of T cells by blocking glycosphingolipid synthesis in SK-RC-45 cells (11), we extended these studies by showing that gangliosides isolated from both SK-RC-54V and SK-RC-26B cell lines could induce death in T cells. SK-RC-54V gangliosides induced ~50% of T cells to undergo apoptosis as evidenced by Annexin V/7-AAD-positive staining of the T cells (Fig. 1B). Similarly, SK-RC-26B-derived gangliosides displayed potent T-cell killing activity, with ~30% T-cell death at 4 μg of total LSA (Fig. 1C). Measuring total LSA provided an estimate of the ganglioside concentration expressed by the RCC cell lines because the majority of sialic acid bound to lipid represents gangliosides (31).

**GM2 expressed by RCC cell lines contributes to tumor-induced T-cell apoptosis.** To establish that GM2 is involved in RCC-induced apoptosis of T cells, we first examined RCC lines for GM2 expression. HPLC analysis of the gangliosides isolated from SK-RC-54V cells and SK-RC-26B cell lines shows expression of GM2 (Fig. 2A) along with several other gangliosides. Furthermore, HPTLC analysis confirmed that SK-RC-54V cells expressed GM2 because a band migrating with the same Rf value as standard GM2 was shown to be present in the gangliosides isolated from SK-RC-54V cells (data not shown). As seen in Fig. 2B, flow cytometry analysis of RCC lines stained with anti-GM2 antibody showed that SK-RC-54V, SK-RC-26B, and 0827 Lung Met cell lines expressed a significantly higher percentage of GM2-positive cells when compared with the immortalized NKE cell line. These findings show that several RCC lines, which are potent inducers of T-cell apoptosis, also express GM2.

Blocking experiments with anti-GM2 antibody were done to determine whether the GM2 expressed by the RCC lines was at least partially responsible for T-cell apoptosis. T cells were cocultured with SK-RC-54V and SK-RC 26B cells for 72 hours in

---

**Figure 1.** Induction of T-cell death by RCC lines and gangliosides derived from these cell lines. Three RCC lines were grown for 96 hours to confluence, at which time half of the medium was replaced by peripheral blood T cells (1 x 10⁶/mL) and cocultured for 72 hours. A, nonadherent T cells were removed from the adherent tumor monolayers, and the number of dead/live cells was determined by trypan blue staining. The mean level of T-cell death induced by the three RCC cell lines was significantly increased over the degree of apoptosis induced by controls, medium alone, and NKE. *, P < 0.001 versus control. B, T cells (1 x 10⁶/mL) were treated with SK-RC-54V gangliosides for 72 hours. T cells were then stained with anti-CD3 antibody, Annexin V, and 7-AAD. Events (10 x 10⁴) were acquired on a FACScan followed by flow cytometric analysis using quadrant software. C, T cells cocultured with SK-RC-26B gangliosides at different concentrations were assayed by the trypan blue exclusion method.
the presence or absence of the anti-GM2 antibody. Representative
data (Fig. 2C) show that, although SK-RC-54V cells (Fig. 2C, top)
duces DNA breaks in T cells (65%, TUNEL positive), the addition
of anti-GM2 antibody to the cocultures caused a 50% reduction of
lymphocyte apoptosis. Similarly, SK-RC-26B cells caused 30% of
T cells to undergo cell death, whereas the inclusion of anti-GM2
antibody to the cultures caused >50% inhibition in tumor-induced
apoptosis (Fig. 2C, bottom), suggesting that GM2 plays a role in
tumor cell killing of T cells. Normal hamster IgG used as a negative
control had no inhibitory effect on the apoptogenic activity of
either SK-RC-54V or SK-RC-26B cells. The specificity of the GM2
antibody has been shown using an ELISA-based assay, which
showed that anti-GM2 (DMF10.167.4) antibody recognized GM2
but not other gangliosides (27). Our immunostaining of TLC plates

Figure 2. GM2 expressed by RCC lines contributes to tumor-induced T-cell apoptosis. A, HPLC profiles of gangliosides isolated from SK-RC-54V and SK-RC-26B
cell lines were obtained as described previously in Materials and Methods. B, cell lines (2 × 10⁶ per tube) were stained with anti-GM2 antibody or IgG isotype control for
30 minutes at 4°C. T cells were then incubated with FITC-conjugated goat anti-hamster IgG antibody, and at least 20,000 events were acquired by FACScan.
Percentage of GM2-positive cells in RCC cell lines SK-RC-54V, SK-RC-26B, and 0827 Lung Met (0827LM) and the normal epithelial cell line NKE. Similar results were
observed in two more experiments. C, RCC cell lines SK-RC-54V (top) and SK-RC-26B (bottom) grown to confluence for 4 to 5 days were pretreated for 3 hours
with either anti-GM2 antibody (GM2 Ab; 1 μg/mL) or control IgG antibody (IgG Ab; 1 μg/mL) before adding T cells. Following coculture for 72 hours, T cells were
harvested for either TUNEL assay or trypan blue exclusion. The mean increase in T-cell death was significantly greater for lymphocytes cultured with tumor cells
(with or without IgG control) versus medium. *, P < 0.03 versus T cells + medium. Addition of anti-GM2 antibody significantly reduced tumor-induced T-cell death.
**, P < 0.02 versus T cells + SK-RC-26B (n = 3). D, specificity of the hamster anti-human GM2 antibody (IgG) was determined by immunostaining of HPTLC
plates spotted with a mixture of purified bovine brain-derived gangliosides: GM3, GM2, GM1, and GD3 (lane 1), purified GM2 (lane 2), and a mixture containing
no GM2 (GM3, GM1, and GD3; lane 3). Plates were incubated with anti-GM2 antibody followed by counterstaining with secondary antibody (biotin-conjugated goat
anti-hamster IgG) for 1 hour as described in Materials and Methods.
also confirmed the specificity (Fig. 2 D) because the antibody reacts specifically with the mixture of bovine brain-derived gangliosides that contained GM2 and with purified GM2 alone but not with the ganglioside mixture devoid of GM2 (GM3, GM1, and GD3). Protection against RCC cell line-induced T-cell death was also observed using the mouse anti-GM2 antibody KM696 (IgM; gift from Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan; data not shown). We also showed that neither mouse anti-GD3 (IgG) nor mouse anti-GD2 (IgG) antibodies blocked any T-cell death induced by the RCC cell lines, which express both GD2 and GD3 (data not shown).

At the concentrations used, the anti-GM2 antibody did not affect the viability of the tumor monolayers (data not shown), ruling out the possibility that the reduction in T-cell death was due to a reduction in either the viability or number of tumors in the plates. Therefore, the reduction in T-cell apoptosis induced by anti-GM2 antibody seems to be attributable to the anti-GM2 antibody interfering with the ability of the GM2 on the tumor cells to interact with the T lymphocytes.

**GM2 is highly expressed in RCC tumors.** Here, we examined the ganglioside composition expressed primarily by clear cell RCC tumors because this is the most common form of sporadic kidney cancer (17 clear cell and 1 chromophobe; n = 18). The HPLC elution profiles and HPTLC migration patterns of gangliosides isolated from RCC tissues were compared with that of a mixture of standard gangliosides run in the same experiments. The most frequently expressed gangliosides by clear cell RCCs included those with retention times consistent with GM3 (50%), GM2 (83%), GM1 (33%), and GD1a (39%) using HPLC (Fig. 3 B), with similar results noted for HPTLC analysis. GD1b and GT1b gangliosides were infrequently expressed in RCC tissues (GD1b, 11% and GT1b, 6%). Mass spectrometry (MS) analysis (liquid chromatography electrospray ionization tandem MS) confirmed that GM3, GM2, GM1, and GD1a were the gangliosides most frequently expressed on clear cell RCC (data not shown). Figure 3 displays representative HPLC profiles of two RCC tissue-derived gangliosides, showing significant GM2 expression in tumor. HPTLC analysis of RCC-derived
gangliosides also show appreciable expression of GM2 (Fig. 3C). Finally, immunostaining of short-term cell lines derived from RCC tissues with anti-GM2 antibody shows GM2 expression as evidenced by green fluorescence in confocal microscopy (Fig. 3D). These findings suggest that GM2 is not only expressed by RCC cell lines but also expressed in RCC tissues.

**RCC tissue-derived gangliosides induce MPT and T-cell death.** Because gangliosides derived from RCC lines were apoptogenic for T cells, we also tested whether gangliosides isolated from tumor tissue of patients with clear RCC were also apoptogenic. Here, we show that, when T cells were cultured with RCC tissue-derived gangliosides (n = 6), a significant percentage (23–48%) of the lymphocytes were induced to undergo cell death compared with controls (Fig. 4A). Similar results were obtained with gangliosides isolated from five additional tumors (data not shown). The concentration of ganglioside that induced apoptosis ranged between 4 and 20 μg/mL.

We also tested whether gangliosides induce mitochondrial damage in T cells as a potential mechanism of mediating T-cell death. To address this question, T cells were treated with RCC-derived gangliosides for 72 hours followed by staining with DiOC6, a selective dye for mitochondria. When compared with untreated T cells, those incubated with gangliosides showed a reduced uptake of DiOC6, suggesting that RCC-derived gangliosides can induce MPT in lymphocytes (Fig. 4B). Similar findings were observed with gangliosides isolated from two additional RCC tissues (data not shown).

**RCC tissue-derived ganglioside-induced T-cell death is partly mediated by GM2.** To determine whether GM2 contributed to the T-cell killing induced by RCC-derived gangliosides, lymphocytes were incubated with gangliosides isolated from three different clear cell RCCs in the presence and absence of anti-GM2 antibody (DMF10.167.4). Two of the isolated gangliosides expressed GM2 (RCC-16 and RCC-3), whereas a third was deficient in GM2 as determined by HPLC and MS (RCC-34). Gangliosides induced 52% to 58% of the cocultured T cells to die. The addition of anti-GM2 antibody significantly blocked T-cell killing mediated by the two RCC-derived ganglioside preparations that expressed GM2 (52% reduction for RCC 16 and 44.6% reduction for RCC 3; Fig. 4C). Nonimmune isotype control IgG on the other hand did not block T-cell death (Fig. 4C). As anticipated, the apoptogenic activity of the ganglioside preparation not expressing GM2 was unaffected by the addition of the anti-GM2 antibody (Fig. 4C). These findings suggest that GM2 as well as additional RCC tissue-derived gangliosides are apoptogenic for T cells.

**RCC tissue-derived gangliosides, including GM2, suppress Th1 and Th2 response in CD4+ T cells.** We also tested whether the RCC-derived gangliosides would regulate the expression of a Th1 and/or Th2 cytokine response *in vitro* because our previous study suggested that there was a diminished Th1 response in tumor-infiltrating lymphocytes and in the peripheral blood of RCC patients (32, 33). T cells were incubated with gangliosides isolated from RCC tissue or the RCC line SK-RC-26B for 24 hours before stimulating the lymphocytes with cross-linked anti-CD3/anti-CD28 antibodies and analyzing cells for their intracellular expression of IFN-γ and IL-4. The majority of the intracellular IFN-γ and IL-4 expression following stimulation was observed in the CD4+ T-cell population (Fig. 5A). Gangliosides isolated from six different RCC tumors all significantly suppressed IFN-γ production (mean, 73% inhibition for CD3 and 62% for CD4). Inhibition of IL-4 expression was more variably suppressed by gangliosides. Four of six gangliosides were inhibitory for IL-4 expression (mean, 65% inhibition for CD3 and 67% for CD4; n = 4), whereas two had no effect (Fig. 5A and B). Similar results were observed with gangliosides isolated...
Cancer Research 2006; 66: (13). July 1, 2006 6822 www.aacrjournals.org

Discussion

Numerous reports have shown increased ganglioside expression in various histologic types of tumors, including RCC, which likely results from altered expression of enzymes regulating ganglioside synthesis (16–18, 34–36). Several groups have reported significantly higher levels of gangliosides in plasma and serum of patients with various forms of cancers compared with healthy volunteers, where the elevated levels of gangliosides correlated with poor clinical outcome (18, 37). Additionally, tumor-derived gangliosides can inhibit development of an antitumor antigen response in vivo in several different murine studies (38, 39). Although the functional role of GM2 has mainly centered on its effect on the tumor cells, GM2 can have a negative effect on the development of an immune response. Bovine brain-derived GM2 suppressed dendritic cell maturation and function (40), the proliferative response of human T cells to tetanus toxoid, and induced apoptosis of a cytotoxic mouse T-cell line, CTLL-2 (22, 41).

In this study, we examined the effect of human tumor-derived gangliosides in T-cell apoptosis. The RCC-derived gangliosides were substantially more potent at inducing apoptosis in peripheral T cells than the purified bovine brain-derived gangliosides. Significant apoptosis (>30%) induced by RCC tumor-derived gangliosides was observed at 4 to 20 µg/mL (data not shown), whereas purified bovine brain-derived gangliosides, when used alone, only induced apoptosis at concentrations >150 µg/mL (8). The greater apoptotic activity of the RCC-derived gangliosides might be partly

from the tumor line SK-RC-26B (84% inhibition of IFN-γ and 44% suppression of IL-4). Dose-response curves showed that these gangliosides could effectively suppress IFN-γ production at concentrations of 1 ng/mL, with significant activity even at 100 pg/mL (Fig. 5C). Additional experiments showed that GM2 present in the ganglioside preparations contributed to the suppression of IFN-γ. The addition of anti-GM2 antibody to T cells partially blocked IFN-γ suppression mediated by the gangliosides, whereas isotype control IgG had no blocking effect (Fig. 5D). To further substantiate a role for GM2 in suppressing a Th1 response, T cells were cultured with purified bovine brain-derived GM2 for 24 hours before stimulating with anti-CD3/anti-CD28 antibodies (or PMA/ionomycin) and assessing CD4+ and CD8+ T-cell subsets for intracellular expression of IFN-γ as well as IL-4. Interestingly, bovine brain-derived GM2 inhibited IFN-γ production at very low concentrations (10 ng; Fig. 6A and C); however, it had only minimal effect on the intracellular expression of IL-4 (Fig. 6B), suggesting that it preferentially suppresses a Th1 but not a Th2 response.

Figure 5. RCC-derived gangliosides suppress both Th1 and Th2 type 1 responses. T cells were activated (anti-CD3/anti-CD28 antibodies) in the presence or absence of different concentrations of RCC tissue-derived gangliosides before measuring intracellular levels of cytokines by flow cytometry. A, suppressive effect of six different RCC tissue-derived gangliosides (1 µg/mL) on intracellular expression of IFN-γ by all T cells as well as the CD4+ subset. The mean level of intracellular IFN-γ in both CD3 and CD4 subsets is significantly reduced in T cells cultured with RCC gangliosides versus medium (*, P < 0.01 versus medium; **, P < 0.02 versus medium) but that for the CD4 subset was not significant because two of six RCC gangliosides actually increased IL-4 expression (**, P < 0.6 versus medium). The level of suppression for each ganglioside is as follows: CD3, 11.10%, 34.24%, 75.78%, 63.89%, 80.37%, and 66.18% versus medium and CD4, −63.97%, −36.44%, 26.72%, 78.58%, 87.78%, and 79.23% versus medium. C, T cells were cultured with three RCC tissue-derived gangliosides for 24 hours before stimulation with anti-CD3/anti-CD28 antibodies. T cells were stained with anti-CD4 antibody, and intracellular staining for IFN-γ was done. Representative data from one of two experiments. D, T cells were cultured with RCC 35 ganglioside for 24 hours in the presence or absence of anti-GM2 antibody (or control IgG) at 1 µg/mL followed by stimulation or not with PMA/ionomycin. Cells were surface stained with anti-CD4 antibody, and intracellular staining for IFN-γ was done. The suppression of intracellular IFN-γ in T cells cultured with RCC 35 ganglioside (with or without IgG control) is significantly increased versus medium (*, P < 0.01 versus medium; ***, P < 0.04 versus medium (n = 3 experiments). Addition of anti-GM2 antibody significantly reverses the phenotype. **, P < 0.05 versus PMA/ionomycin + RCC 35 ganglioside.

from the tumor line SK-RC-26B (84% inhibition of IFN-γ and 44% suppression of IL-4). Dose-response curves showed that these gangliosides could effectively suppress IFN-γ production at concentrations of 1 ng/mL, with significant activity even at 100 pg/mL (Fig. 5C). Additional experiments showed that GM2 present in the ganglioside preparations contributed to the suppression of IFN-γ. The addition of anti-GM2 antibody to T cells partially blocked IFN-γ suppression mediated by the gangliosides, whereas isotype control IgG had no blocking effect (Fig. 5D). To further substantiate a role for GM2 in suppressing a Th1 response, T cells were cultured with purified bovine brain-derived GM2 for 24 hours before stimulating with anti-CD3/anti-CD28 antibodies (or PMA/ionomycin) and assessing CD4+ and CD8+ T-cell subsets for intracellular expression of IFN-γ as well as IL-4. Interestingly, bovine brain-derived GM2 inhibited IFN-γ production at very low concentrations (10 ng; Fig. 6A and C); however, it had only minimal effect on the intracellular expression of IL-4 (Fig. 6B), suggesting that it preferentially suppresses a Th1 but not a Th2 response.

Figure 5. RCC-derived gangliosides suppress both Th1 and Th2 type 1 responses. T cells were activated (anti-CD3/anti-CD28 antibodies) in the presence or absence of different concentrations of RCC tissue-derived gangliosides before measuring intracellular levels of cytokines by flow cytometry. A, suppressive effect of six different RCC tissue-derived gangliosides (1 µg/mL) on intracellular expression of IFN-γ by all T cells as well as the CD4+ subset. The mean level of intracellular IFN-γ in both CD3 and CD4 subsets is significantly reduced in T cells cultured with RCC gangliosides versus medium (*, P < 0.01 versus medium; **, P < 0.02 versus medium) but that for the CD4 subset was not significant because two of six RCC gangliosides actually increased IL-4 expression (**, P < 0.6 versus medium). The level of suppression for each ganglioside is as follows: CD3, 11.10%, 34.24%, 75.78%, 63.89%, 80.37%, and 66.18% versus medium and CD4, −63.97%, −36.44%, 26.72%, 78.58%, 87.78%, and 79.23% versus medium. C, T cells were cultured with three RCC tissue-derived gangliosides for 24 hours before stimulation with anti-CD3/anti-CD28 antibodies. T cells were stained with anti-CD4 antibody, and intracellular staining for IFN-γ was done. Representative data from one of two experiments. D, T cells were cultured with RCC 35 ganglioside for 24 hours in the presence or absence of anti-GM2 antibody (or control IgG) at 1 µg/mL followed by stimulation or not with PMA/ionomycin. Cells were surface stained with anti-CD4 antibody, and intracellular staining for IFN-γ was done. The suppression of intracellular IFN-γ in T cells cultured with RCC 35 ganglioside (with or without IgG control) is significantly increased versus medium (*, P < 0.01 versus medium; ***, P < 0.04 versus medium (n = 3 experiments). Addition of anti-GM2 antibody significantly reverses the phenotype. **, P < 0.05 versus PMA/ionomycin + RCC 35 ganglioside.
attributable to modifications in the structure of tumor-derived gangliosides (39).

Our findings suggest that GM2 has a role in the T-cell dysfunction observed in RCC patients. T-cell death induced by either RCC tissue-derived gangliosides or RCC cell lines was partially blocked by anti-GM2 antibody. We have also found that supernatants from RCC explants can induce T-cell apoptosis and that gangliosides isolated from the tumor supernatants mediate ~60% of the apoptogenic activity within the supernatants. Moreover, immunoprecipitation of the tumor supernatants with anti-GM2 antibody, but not control IgG, significantly reduced the apoptogenic activity of the tumor supernatants. Although GM2 is partly responsible for the apoptosis induced by RCC-derived gangliosides, it seems from our findings that other gangliosides are also involved because the addition of anti-GM2 antibody to T-cell cultures did not completely inhibit the ability of ganglioside preparations expressing GM2 to induce T-cell apoptosis. Furthermore, gangliosides isolated from one RCC tissue, which did not express GM2, were also apoptogenic to T cells.

The mechanism by which gangliosides induce apoptosis is not well defined, with the exception of bovine brain-derived GD3 (42). Here, we show that gangliosides isolated from RCC also induce MPT, as shown by the inability of DiOC$_6$ to localize to the mitochondria in T cells exposed to gangliosides. Additional studies are under way to more precisely define how the RCC-derived gangliosides, including GM2, initiate cell death. The ability of RCC-derived gangliosides to suppress NF-$\kappa$B activation, an apoptotic transcription factor in T cells and dendritic cells, may be a contributing component to ganglioside-induced T-cell death (10, 11, 23, 40, 41).

Tumor-derived gangliosides may also promote T-cell dysfunction in RCC patients by suppressing Th1 and Th2 cytokine responses. A type 1 response (Th1/Th1) plays a critical role in the rejection of tumors (43). Th1-type CD4$^+$ T cells secrete IFN-$\gamma$ that promote cellular immunity, in part by providing helper signals for the cytotoxic CD8$^+$ T lymphocytes (43). Th2-type cells produce IL-4 and IL-5 and typically promote a humoral immune response (44). The issue of type 1 versus type 2 response in RCC patients has been examined, and most studies have indicated that the cytokine profile is most consistent with a type 2 bias in situ (33, 45). Recently, we showed that supernatants from RCC explants as well as gangliosides isolated from the tumor supernatants suppress a Th1 response (IFN-$\gamma$) without inhibiting a Th2 response (IL-5; ref. 46). However, the addition of additional gangliosides from RCC supernatants as well as those from RCC tissues (Fig. 5) showed that gangliosides from some patients can suppress both a Th1 and a Th2 cytokine response, whereas gangliosides from other tumors suppressed only a Th1 response. Interestingly, purified bovine brain-derived gangliosides, including GD1a and GM2, suppress a Th1 response without affecting production of Th2 cytokines (Fig. 6; ref. 46). Other gangliosides, such as GD1b, GT1b, and GQ1b, enhance IL-2 and IFN-$\gamma$ production and suppress IL-4 and IL-5 production (47). Thus, the ganglioside composition expressed by different tumors likely determines whether there is suppression of a Th1 response only, suppression of both Th1 and Th2 responses, or augmentation of a Th2 response. Our neutralizing studies with anti-GM2 antibody identified GM2 as one of the gangliosides

Figure 6. Purified bovine brain-derived GM2 suppresses a Th1 but not a Th2 response. T cells were incubated with 5 $\mu$g/ml purified bovine brain-derived GM2 for 24 hours before stimulation or not with cross-linked anti-CD3 and anti-CD28 antibodies for an additional 48 hours. Thereafter, the cells were fixed, permeabilized, and subsequently stained with antibodies to either IFN-$\gamma$ (A) or IL-4 (B). Aliquots of cells were then stained with antibodies to CD3, CD4, or CD8. A and B, experiments were done twice. Representative data. C, concentrations of GM2 at 10 $\mu$g to 10 ng. Columns, mean of three experiments; bars, SE. Concentrations of 1 and 100 ng were done once. C, T cells incubated with different concentrations of bovine brain-derived GM2 for 24 hours before stimulation with PMA/ionomycin for 48 hours. Cells were then stained with anti-CD4 antibody, and intracellular IFN-$\gamma$ was detected with anti-IFN-$\gamma$ antibody. The suppression of intracellular IFN-$\gamma$ is significant in T cells (CD3$^+$) cultured with 10 $\mu$g to 10 ng purified bovine brain-derived GM2 versus medium. *, P < 0.02; **, P < 0.005; ***, P < 0.01.

---

8 G.S. Sa et al., submitted for publication.
present in RCC tissue and RCC lines that can suppress a Th1 cytokine response.

Recently, we showed that GM2 is involved in T-cell dysfunction in vivo. We find that a significant number of T cells infiltrating RCC stain for GM2 (median, 36% CD3+/GM2+ cells = 12). Although to a lesser degree, whereas peripheral blood T cells from RCC patients stain for GM2 (median, 13% CD3+/GM2+ cells = 18), T cells from healthy volunteers do not (n = 9). Most interestingly, flow cytometry analysis showed that the majority of GM2-positive T cells derived from RCC patients were undergoing apoptosis compared with the GM2-negative population, suggesting that GM2 expression on patient T cells is associated with apoptosis. Overall, our findings suggest that GM2 expressed by RCC represents one mechanism by which tumor cells can evade the immune system.

Gangliosides, including GM2, have been targets for therapy using monoclonal antibodies (mAb) or vaccines to induce anti-GM2 antibodies. A chimeric anti-ganglioside antibody to GM2, KM966 showed antitumor activity against GM2-expressing tumor cells in vitro and also markedly suppressed the establishment of human tumor xenografts in nude mice (48). Recently, a mAb DMF10.167.4 prevented murine lymphoma, human melanoma, and small cell lung carcinoma from establishing tumors in vivo and blocked progression of tumors in vivo (27). Induction of antibodies against GM2 in clinical studies was associated with a better prognosis, but the overall response rate was low (49). Our findings presented here show that, in vitro, anti-GM2 antibodies can partially protect T cells from immune dysfunction induced by tumor-derived GM2. Thus, anti-GM2 antibodies may not only kill tumor cells but may also prevent GM2-positive tumor cells from inducing immune dysfunction.

Acknowledgments

Received 1/23/2006; revised 3/28/2006; accepted 4/7/2006.

Grant support: NIH grants RO1-CA56937 and CA90995 and Kidney Cancer Association Senior Investigator Award.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

25. Retter MW, Johnson JC, Peckham DW, et al. Retinoblastoma-specific antigens expressed by human retinoblastoma tumor xenografts in nude mice (48). Recently, a mAb DMF10.167.4 prevented murine lymphoma, human melanoma, and small cell lung carcinoma from establishing tumors in vivo and blocked progression of tumors in vivo (27). Induction of antibodies against GM2 in clinical studies was associated with a better prognosis, but the overall response rate was low (49). Our findings presented here show that, in vitro, anti-GM2 antibodies can partially protect T cells from immune dysfunction induced by tumor-derived GM2. Thus, anti-GM2 antibodies may not only kill tumor cells but may also prevent GM2-positive tumor cells from inducing immune dysfunction.

Acknowledgments

Received 1/23/2006; revised 3/28/2006; accepted 4/7/2006.

Grant support: NIH grants RO1-CA56937 and CA90995 and Kidney Cancer Association Senior Investigator Award.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
47. Kanda N, Watanabe S. Gangliosides GD1b, GT1b, and GQ1b enhance IL-2 and IFN-γ production and suppress IL-4 and IL-5 production in phytohemagglutinin-stimulated human T cells. J Immunol 2001;166:72–80.
GM2 Expression in Renal Cell Carcinoma: Potential Role in Tumor-Induced T-Cell Dysfunction

Kaushik Biswas, Amy Richmond, Patricia Rayman, et al.


Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/66/13/6816

Cited articles This article cites 48 articles, 19 of which you can access for free at: http://cancerres.aacrjournals.org/content/66/13/6816.full.html#ref-list-1

Citing articles This article has been cited by 12 HighWire-hosted articles. Access the articles at: /content/66/13/6816.full.html#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.