Renal cell carcinoma–derived gangliosides suppress nuclear factor-κB activation in T cells

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Activation of the transcription factor nuclear factor-κB (NFκB) is impaired in T cells from patients with renal cell carcinomas (RCCs). In circulating T cells from a subset of patients with RCCs, the suppression of NFκB binding activity is downstream from the stimulus-induced degradation of the cytoplasmic factor IκBα. Tumor-derived soluble products from cultured RCC explants inhibit NFκB activity in T cells from healthy volunteers, despite a normal level of stimulus-induced IκBα degradation in these cells. The inhibitory agent has several features characteristic of a ganglioside, including sensitivity to neuraminidase but not protease treatment; hydrophobicity; and molecular weight less than 3 kDa. Indeed, we detected gangliosides in supernatants from RCC explants and not from adjacent normal kidney tissue. Gangliosides prepared from RCC supernatants, as well as the purified bovine gangliosides G_{m1} and G_{d1a}, suppressed NFκB binding activity in T cells and reduced expression of the cytokines IL-2 and IFN-γ. Taken together, our findings suggest that tumor-derived gangliosides may blunt antitumor immune responses in patients with RCCs.

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Introduction

Functional T cells are central to an intact antitumor response. However, in patients with renal cell carcinoma (RCCs), tumor progression occurs despite the presence of a substantial lymphoid infiltrate and clones capable of preferentially recognizing tumor antigen (1–4). Effective antitumor immunity depends on the ability of T lymphocytes to undergo normal activation and clonal expansion. In tumor-bearing patients, this fails to develop, as evidenced by diminished capacity of infiltrating T cells to proliferate (5), mediate cytotoxic activity (6), and generate a Th1-type cytokine response in the tumor bed (7). Similar findings have been confirmed in various tumor types in which reduction in delayed-type hypersensitivity and other effector functions of T cells have been noted in animals and patients with progressing tumors (8).

Alterations in select signal-transduction pathways have been associated with immune dysfunction in tumor-bearing hosts (9). Stimulus-dependent activation of the transcription factor nuclear factor-κB (NFκB) has been shown to be impaired in murine tumor models and in patients with kidney cancer (10, 11). The primary defect is failure of the transactivating complex RelA/NFκB1 (p50) to accumulate normally in the nucleus after T-cell activation. Impaired NFκB activity may contribute to reduced T-cell function, as this transcription factor controls the expression of a number of genes that encode for cytokines, their receptors, and other membrane regulatory molecules central to T-cell activation (12–14). This is supported by the finding that a loss of NFκB binding activity in mice with progressing tumors coincides with decreased IL-2 and IFN-γ production.

The tumor itself may play a significant role in the development of immune dysfunction (11, 15–18). In a cohort of more than 80 patients with localized and advanced RCCs, defective activation of κB binding was observed in T cells from nearly 65% of patients, compared with only 6% of 53 age-matched healthy controls (11). Normal NFκB activation was obtained in T cells from nearly 40% of these patients after surgical removal of the tumor (11). In addition, supernatant from explants of tumor, but not from normal kidney, inhibited the activation of κB-specific binding activity in normal T cells, thus mimicking the defect observed in patient-derived T cells (11, 19). Recent findings suggest that at least 2 distinct mechanisms are responsible for the defect in NFκB activation. In 1 set of patients, impaired κB binding activity was attributable to a defect in phosphorylation and degradation of the inhibitor IκBα, and could be reproduced by soluble products from RCCs (19).

In the present report, we describe a second distinct cohort of patients with RCCs whose T-cell NFκB activation is defective despite normal stimulus-dependent degradation of IκBα. This defective state could also be induced in normal T cells after exposure to supernatants prepared from RCC explants in culture. The properties of the soluble product isolated from crude tumor supernatant suggest that gangliosides shed from tumor cells

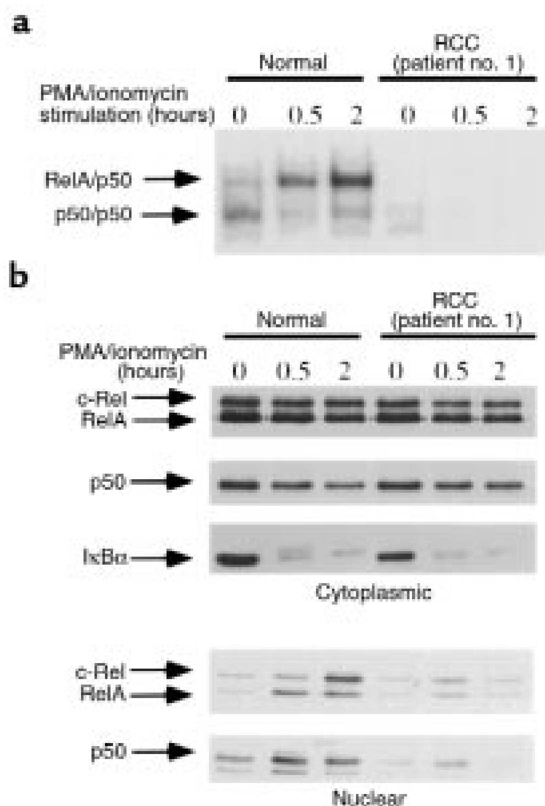

Figure 1

In a cohort of patients with RCCs, there is defective NFκB activation in the presence of normal IκBα degradation. (**a**) Peripheral blood T cells from patients with RCCs were incubated with and without PMA (10 ng/mL) and ionomycin (0.75 μg/mL) for 0, 0.5, and 2 hours. Nuclear extracts were obtained, and EMSA assay was performed with the κB probe (**b**). The nuclear and cytoplasmic extracts from the same sample shown in **a** were analyzed by immunoblotting for RelA, c-Rel, p50, and IκBα expression. Similar results were observed with 9 additional patients.

are responsible for this particular defect. Specifically, the inhibitory product is of low molecular weight, water soluble, and sensitive to neuraminidase, but is resistant to protease. Furthermore, gangliosides were detected in supernatant derived from RCCs, and the inhibitory activity copurified with the isolated gangliosides. These findings suggest that tumor-derived gangliosides play an important role in suppressing the development of T-cell antitumor immunity in patients with renal cancer.

Methods

Reagents. Antibodies to NFκB1 (p50), c-Rel, RelA (p65), and IκBα/MAD-3 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA) and used at 1.5 μg/mL. The secondary antibody was horseradish peroxidase–conjugated donkey anti-rabbit IgG (Amersham Life Sciences Inc., Arlington Heights, Illinois, USA; 1:2,000 dilution in Tris-buffered saline Tween). Antibodies used in magnetic T-cell separation were bead-conjugated monoclonal anti-human CD14 (macrophages), CD16 (NK cells), CD19 (B cells), CD56 (NK cells), and glycophorin A (red blood cells [RBCs]) (Stem Cell Technologies, Vancouver, British Columbia, Canada). PMA (10 ng/mL) and ionomycin (0.75 μg/mL) were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA).

Preparation of RCC explant supernatant. Redundant tissue was obtained from patients undergoing nephrectomy for RCCs at the Cleveland Clinic Foundation. All tissues were obtained from the primary renal lesion (n = 14) or the adjacent normal kidney (n = 8). Tumor supernatants were derived as described previously (19). Briefly, 1 g of tumor explants (3 × 3 mm) was cultured in a T-75 flask with 15 mL of DMEM without additional supplements for 3–4 days at 37°C with 95% O_2/5% CO_2. Supernatant fluid was then filtered, centrifuged, and stored at –70°C. Supernatant from nonneoplastic normal kidney was prepared under identical conditions. The presence of metabolically active cells in the explants was demonstrated by measuring dehydrogenase activity (Colorimetric assay kit; Roche Molecular Biochemicals, Indianapolis, Indiana, USA).

T-cell isolation. Lymphocytes were obtained from healthy volunteers who had been leukophoresed (n = 25). Peripheral blood lymphocytes (PBLs) were centrifuged over a Ficoll-Hypaque density gradient (LKD Biotech, Piscataway, New Jersey, USA), and T cells were purified by negative magnetic selection using microbeads coated with antibodies to CD14 (macrophages), CD16 (NK cells), CD19 (B cells), CD56 (NK cells), and glycophorin A

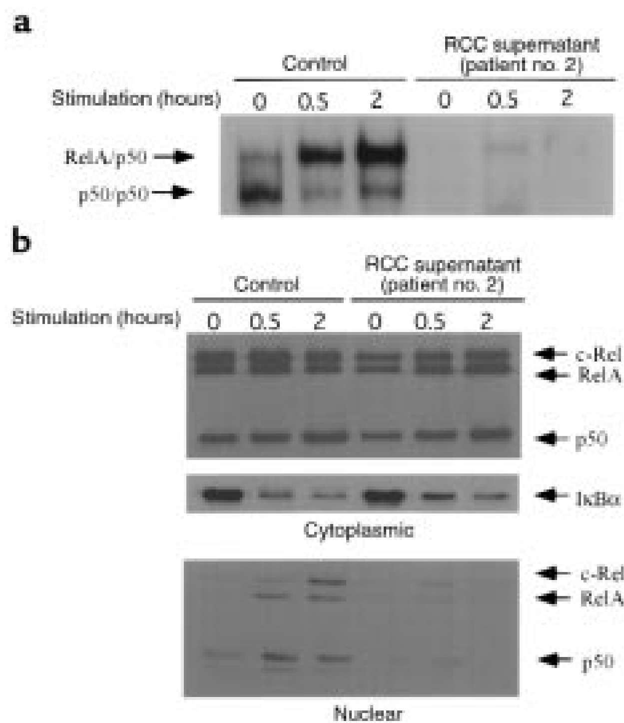

Figure 2

Soluble products from RCC explants suppress NFκB activation in normal T cells. (**a**) Peripheral blood T cells from healthy donors were incubated with medium or medium supplemented with tumor supernatant for 18 hours, and were then stimulated with PMA (10 ng/mL) and ionomycin (0.75 μg/mL) for 0, 0.5, and 2 hours. Nuclear extracts were isolated, and EMSA assay performed with the κB probe (**b**). The nuclear and cytoplasmic extracts from the same sample shown in **a** were analyzed by immunoblotting for RelA, c-Rel, p50, and IκBα. Similar results were observed with supernatant from 7 additional tumor specimens.

(RBCs). This resulted in a greater than 96% $CD3^+$ cell population, as determined by immunocytometry. This same procedure was used to isolate T cells from the peripheral blood of patients with RCCs ($n = 10$).

Induction of impaired NFκB activation by tumor supernatant and purified gangliosides. T cells were cocultured in complete RPMI-1640 with 2% FBS in the presence or absence of supernatant derived from explants of either renal tumors or uninvolved area of the kidney (normal kidney cell supernatant). The volume of crude supernatant added varied between 30% and 50% of the total volume. In some experiments, T cells were preincubated with gangliosides isolated from neuraminidase-sensitive RCC supernatants or with purified bovine brain gangliosides G_{m1}, G_{m3}, and G_{d1a} (Sigma Chemical Co.) In the case of isolated tumor-derived gangliosides, the concentration of ganglioside added to T-cell cocultures approximated that of the corresponding crude supernatant from which it was derived. Cells were then incubated at 37°C with 5% CO_2 for 18 hours before stimulation with PMA (10 ng/mL) and ionomycin (0.75 μg/mL).

Characterization of suppressive products in RCC supernatant. Supernatant was passed through an Amicon filtration unit loaded with a YM3 Diaflo ultrafiltration membrane (Amicon Inc., Beverly, Massachusetts, USA). The fractions that were less than 3 kDa and more than 3 kDa were then evaluated in T-cell coculture experiments at concentrations equal to those of the corresponding crude supernatant. The hydrophobicity was evaluated using stepwise elution from a C18 Sep-Pak cartridge (Waters Corp., Milford, Massachusetts, USA). Samples were prepared in 15% ethanol/10% formic acid and loaded, and the eluate was collected (fraction 1). Bound material was consecutively eluted with 15% ethanol (fraction 2), petroleum ether (fraction 3), and ethyl acetate (fraction 4). Fractions were titrated to pH 7.0, dried under N_2, and then reconstituted in the appropriate diluent before T-cell coculture experiments. The inhibitory product was also tested for its sensitivity to trypsin (1 μg/mL), proteinase K (50 μg/mL), or neuraminidase (0.1 U/mL) at 37°C for 24 hours.

Electrophoretic mobility shift assays. Nuclear extracts were prepared from T cells before and after stimulation with PMA/ionomycin (0, 0.5, and 2 hours). Cell pellets were suspended in 100 μL of hypotonic buffer A containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 1 mM DTT, 100 μg/mL Pefabloc, 10 μg/mL PMSF, and 0.1 μg/mL chymostatin for 20 minutes on ice. Next, 10 μL of 10% Nonidet P-40 (Sigma Chemical Co.) was added to each sample, which was then vortexed before centrifugation. The cytoplasmic extract was aliquoted, and the nuclear pellet was rinsed with hypotonic buffer A. The nuclear pellet was then resuspended in a higher-salt buffer C containing 20 mM HEPES (pH 7.9), 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 1 mM DTT, 100 μg/mL Pefabloc, 10 μg/mL PMSF, and 0.1 μg/mL chymostatin for 20 minutes at 4°C. Samples were then centrifuged (136 *g*) at 4°C for 10 minutes, and nuclear extracts were aliquoted. Protein concentrations were measured with BCA protein assay reagent (Pierce Chemical Co., Rockford, Illinois, USA), and equal amounts were loaded for electrophoretic mobility shift assay (EMSA).

An oligonucleotide containing a tandem repeat corresponding to the κB element of the IL-2R gene was prepared by annealing a coding strand template to a complementa-

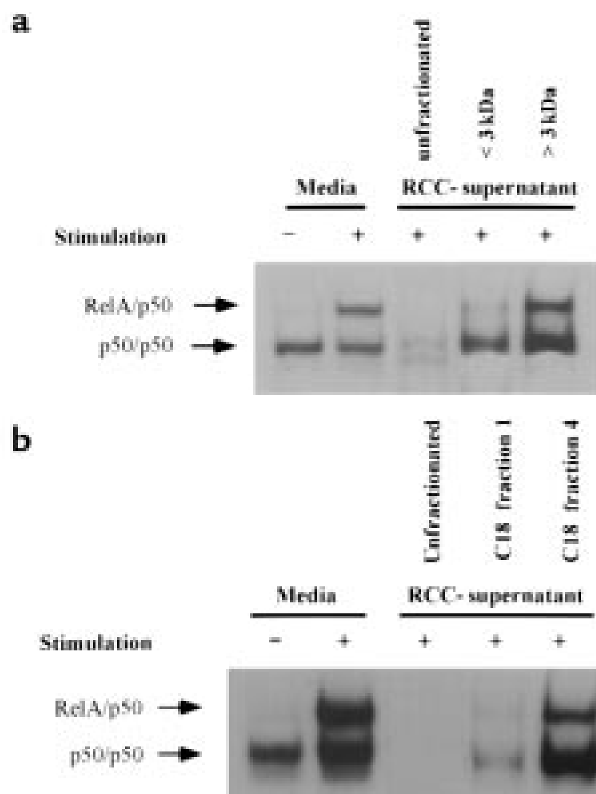

Figure 3

The product in tumor supernatant responsible for inhibiting κB binding activity weighs less than 3 kDa and is water soluble (**a**). Normal T cells were incubated with 1 of the following for 18 hours: media, unfractionated RCC supernatant, or the fractions that were less than 3 kDa or more than 3 kDa after passage through a YM3 Diaflo membrane. T cells were then stimulated for 2 hours with PMA (10 ng/mL) and ionomycin (0.75 μg/mL) before preparation of nuclear extract for EMSA using the κB probe. Similar findings were noted in 7 additional experiments (**b**). The less than 3-kDa fraction of the RCC supernatant was loaded onto a C18 Sep-Pak cartridge. Eluate was collected (fraction 1), and bound material was eluted with 15% ethanol (fraction 2), petroleum ether (fraction 3), and ethyl acetate (fraction 4). T cells were then incubated with medium, unfractionated RCC supernatant, or C18 fractions for 18 hours before stimulation with PMA/ionomycin for 2 hours. Nuclear extracts were harvested, and EMSA was performed. The results with fractions 2 and 3 were not shown because they were similar to those of fraction 4. Similar results were obtained in 3 additional experiments.

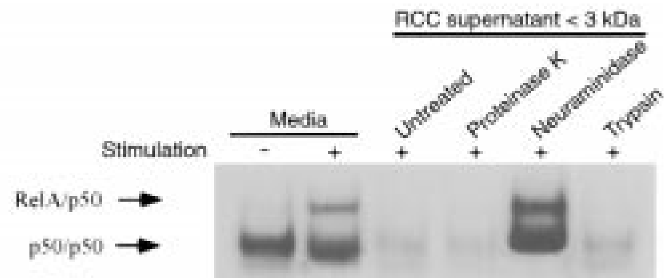

Figure 4
The inhibitory molecule in RCC supernatants is sensitive to neuraminidase. The less than 3-kDa fraction of RCC supernatant was treated with neuraminidase, proteinase K, or trypsin for 24 hours as detailed in Methods. Thereafter, T cells from a healthy donor were incubated with untreated and enzyme-treated RCC supernatant for 18 hours before stimulation with PMA/ionomycin. EMSA was then performed using nuclear extract.

ry 10-base primer, and by filling in the overhang using DNA polymerase I in the presence of [α-^{32}P]dCTP. The sequence was 5′-CAACGGCAGGGGAATCTCCCTCTCCTT-3′. The underlined portion represents the κB binding motif.

Binding reactions were performed using 10 μg of nuclear protein preincubated on ice for 10 minutes in a 25-μL total reaction volume containing 20 mM HEPES (pH 7.9), 80 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 8% glycerol, and 2 μg of poly(dI-dC) (Amersham Pharmacia Biotech Inc., Piscataway, New Jersey, USA). The reaction mixture was then incubated with the radiolabeled oligonucleotide for 20 minutes at room temperature. Samples were analyzed by electrophoresis in a 6% nondenaturing polyacrylamide gel with 0.25 TBE buffer (22.2 mM Tris, 22.2 mM boric acid, 0.5 mM EDTA). Gels were vacuum-dried and exposed to film at –80°C.

Immunoblotting. Cytoplasmic protein samples (10–20 μg) were mixed with an equal amount of 2× Laemmli sample buffer, boiled, and resolved by electrophoresis in 10% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane that was incubated in 5% nonfat dry milk in Tris-buffered saline overnight. Thereafter, membranes were incubated sequentially with specific antibody (2 hours) and then with horseradish peroxidase–conjugated donkey anti-rabbit IgG for 30 minutes. Membranes were then developed with enhanced chemiluminescence (ECL Western Blotting Kit; Amersham Life Sciences).

Isolation of gangliosides from tumor tissue and supernatant derived from RCC explants. Gangliosides were isolated from suppressive supernatants (<3-kDa fraction, neuraminidase sensitive) (n = 7) and normal kidney (n = 3) using previously established methods (20). Initially, lipids were extracted in chloroform/methanol (1:1) for 18 hours, followed by differential partitioning of the lipid extract in diisopropyl ether/1-butanol. The isolated gangliosides were reconstituted in dd-$H_2$0, sonicated, and passed through a Sephadex G-25 column. Isolated gangliosides eluting in the void volume were lyophilized, redissolved in chloroform/methanol, resolved by TLC as described previously (21), and detected using a resorcinol-HCl-Cu^{2+} reagent (22).

Detection of cytokine production. PBLs from healthy volunteers were isolated by Ficoll-Hypaque gradient as already described. Cells (10^6/mL) were preincubated for 48 hours with supernatant (1:1 vol/vol) from RCC explants or isolated gangliosides in either the presence or absence of stimulus (PMA/ionomycin). After incubation, cell supernatants were assayed for expression of IL-2 and IFN-γ by ELISA (Quantikine HS kits; R&D Systems Inc., Minneapolis, Minnesota, USA).

Results

Defective NFκB activation is independent of IκBα degradation. Previously, we reported that in T cells obtained from 1 subset of patients with RCC (n = 7), stimulus-induced IκBα degradation was blocked, preventing the activation of NFκB (19). We now describe a second cohort of patients (n = 10) in whom NFκB is not activated despite normal degradation of IκBα. Representative data depict-

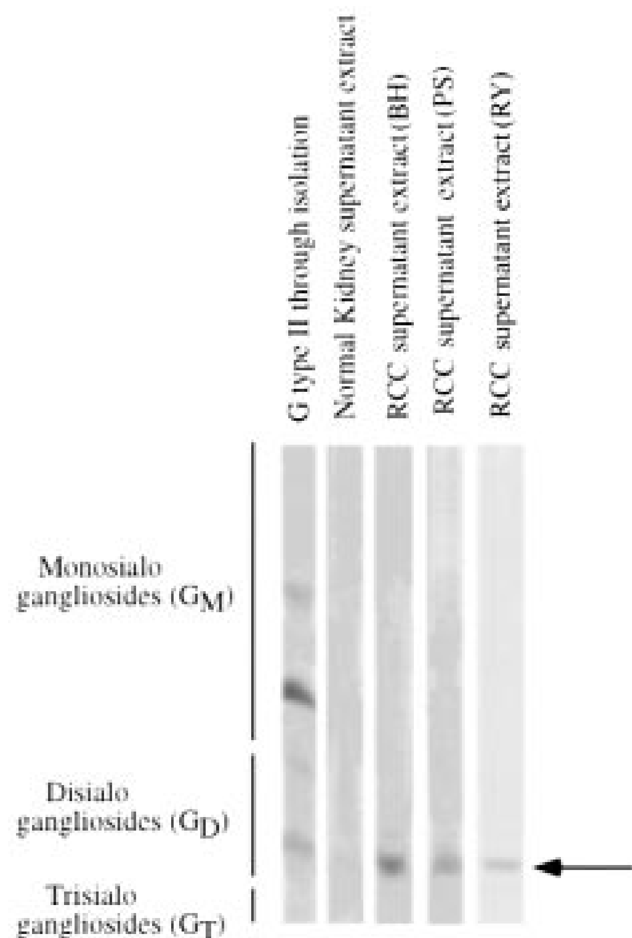

Figure 5
Gangliosides are present in RCC supernatants that suppress NFκB activation. Gangliosides were extracted from supernatants of normal kidney and RCC explants using the procedure outlined in Methods. Aliquots of isolated gangliosides were then subjected to TLC and detected by staining with resorcinol-HCL-Cu^{2+}. As a positive control, G type II ganglioside mixture (bovine gangliosides) was reisolated and run on TLC.

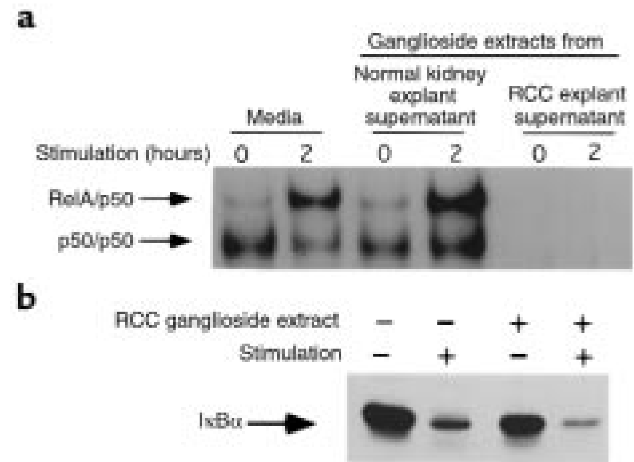

Figure 6
Gangliosides isolated from supernatants of RCC explants inhibited kB binding activity. T cells derived from normal healthy donors were incubated with medium or gangliosides isolated from supernatants of normal kidney explants and RCC explants. After 18 hours, T cells were stimulated with PMA/ionomycin for 2 hours, and kB binding activity of nuclear extract was determined (**a**). Gangliosides were added to the T-cell culture at concentrations equivalent to those used to demonstrate suppression by the corresponding crude supernatant. Western blotting with antibody to IkBα is shown in **b**.

ing this phenotype of NFκB dysfunction are presented in Figure 1, and are compared with normal induction observed in T cells from healthy volunteers. Normal T cells show 2 specific κB binding complexes after stimulation. The slower-migrating band represents the transactivating p50/RelA heterodimer, whereas the faster-migrating complex is a p50 homodimer (23). In patient T cells, stimulation with PMA/ionomycin did not result in κB binding activity of either complex, although the magnitude of IκBα degradation was similar to that observed in T cells from healthy volunteers. The impaired accumulation of Rel proteins (c-Rel, RelA, and p50) in the nucleus was not due to reduced cytoplasmic levels of these proteins, as their levels were comparable in normal and patient T cells (Figure 1).

RCC-derived soluble products inhibit the stimulus-dependent nuclear accumulation of Rel proteins in normal peripheral blood–derived T cells without altering IκBα processing. T cells from normal donors were cultured with and without tumor supernatant for 18 hours before stimulation with PMA/ionomycin and measurement of κB binding activity in isolated nuclei (Figure 2). Incubation of T cells with tumor supernatants inhibited the activation of NFκB binding activity and impaired the nuclear accumulation of Rel proteins (RelA, c-Rel, p50), although cytosolic levels were not different from controls. NFκB activation was suppressed even though normal stimulus-induced degradation of IκBα was observed (Figure 2). Supernatants from normal kidney explants were not suppressive, suggesting that the inhibitory activity was derived from tumor tissue (data not shown).

Characterization of tumor-derived inhibitory activity. The inhibitory activity obtained from 8 separate supernatants passed through an ultrafiltration membrane with a 3,000 molecular weight cutoff, whereas the fraction that weighed more than 3 kDa had no effect (Figure 3a). After acidification, the supernatants were loaded on a C18 Sep-Pak cartridge and subjected to stepwise elution. The inhibitory activity did not bind to the column but, instead, eluted with the aqueous loading buffer (fraction 1; Figure 3b). Based on the elution profile, the inhibitory activity is unrelated to polar and nonpolar lipids (data not shown). Furthermore, the inhibitory activity could be distinguished from arachidonic acid metabolites, which have an affinity for the matrix and elute with ethyl acetate (fraction 4; Figure 3b). Treatment with neuraminidase of the fraction that weighed less than 3 kDa eliminated its suppressive activity, whereas activity was insensitive to proteinase K and to trypsin, both of which had no effect on suppression of NFκB, suggesting that this product is not a protein and contains neuraminic acid (Figure 4). Based on molecular size, the inhibitor is distinct from mucins and larger glycoproteins. Taken together, these properties are consistent with those of a ganglioside.

Gangliosides isolated from tumor supernatants inhibit NFκB activation. To test the hypothesis that the RCC-derived inhibitors of NFκB activation were gangliosides, supernatants were examined for ganglioside content. Ganglioside isolation was performed on previously characterized tumor supernatant specimens in which the suppressive activity was less than 3 kDa, and which were water soluble and neuraminidase sensitive. DMEM spiked with purified bovine gangliosides was used as a control for the isolation procedure. TLC analysis of the isolated prod-

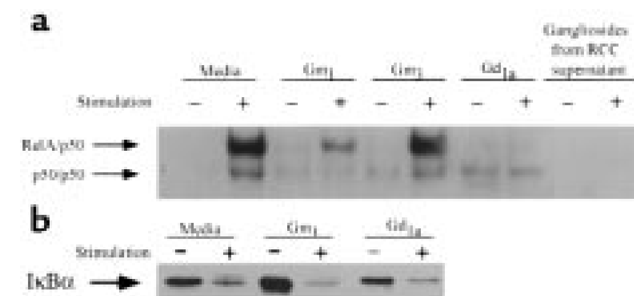

Figure 7
Inhibition of κB binding activity can be induced by ganglioside types that are overexpressed in RCCs. Normal T cells were incubated for 18 hours with bovine brain–derived G_{m1}, G_{m3}, and G_{d1a}, as well as gangliosides isolated from RCC supernatants. Cells were then stimulated for 2 hours with PMA/ionomycin before evaluating κB binding activity of nuclear extracts. κB binding activity is shown in **a**, and IκBα degradation is presented in **b**.

ucts is illustrated in Figure 5 and demonstrates the presence of gangliosides in supernatants from RCCs. The chromatographic pattern was similar for each of the tumor supernatants tested, and, in all cases, the major isoform migrated slightly lower than bovine-derived purified disialoganglioside. The slight difference in migration pattern may reflect differences in the ganglioside structure between disialogangliosides isolated from the tumor and those isolated from normal bovine brain tissue. No gangliosides were detected in supernatant obtained from cultured normal kidney explants.

Preincubation of normal T cells with isolated gangliosides inhibited the stimulation of κB binding activity in response to PMA/ionomycin (Figure 6a). Significant suppression was detected after 12 hours of incubation with tumor-derived gangliosides, and reached maximal inhibition by 24 hours. Material prepared from supernatants of normal kidney contained no detectable ganglioside and did not suppress the stimulation of κB binding activity. Furthermore, a commercial source of gangliosides of the same class isolated from the tumor supernatant (disialogangliosides) also suppressed NFκB activation (Figure 7). Finally, coculture of T cells with purified G_{d1a} and G_{m1}, but not G_{m3}, also inhibited NFκB activation. G_{d1a} and G_{m1} have previously been reported to be overexpressed by RCCs (24). All isolated inhibitory gangliosides blocked the stimulation of κB binding activity without altering the degradation of IκBα. These findings suggest that select gangliosides found in tumor supernatant inhibit T-cell NFκB activation.

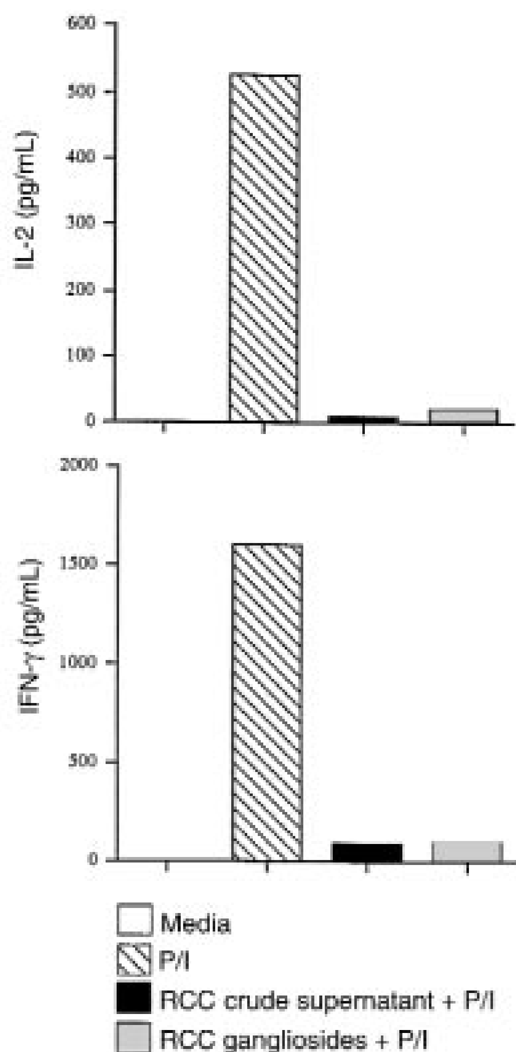

Figure 8
Gangliosides isolated from RCC supernatants inhibit IL-2 and IFN-γ production by PBLs. PBLs from healthy volunteers were preincubated for 48 hours with supernatant (1:1 vol/vol) from RCC explants or isolated gangliosides in either the presence or absence of PMA/ionomycin (P/I). IL-2 and IFN-γ were detected by ELISA. Data from 1 of the 5 representative experiments are presented. In all cases, the inhibition of NFκB by RCC supernatant and derived gangliosides was greater than 90%, relative to control T cells stimulated with PMA/ionomycin.

Ganglioside inhibition of NFκB coincides with reduced production of IL-2 and IFN-γ production. To determine whether inhibitory gangliosides blocked gene expression associated with T-cell activation, we examined the production of IL-2 and IFN-γ by PBLs after exposure to gangliosides prepared from RCC supernatants. IL-2 and IFN-γ secretion stimulated by PMA/ionomycin was inhibited by more than 95% when the T cells had been pretreated with RCC-derived gangliosides (Figure 8). Cytokine levels in the RCC supernatants themselves were negligible (data not shown).

Gangliosides obtained from freshly isolated tumor suppress NFκB activation in T cells. Immunosuppressive gangliosides present in supernatants of RCC explants may represent soluble products shed by the tumor. Gangliosides anchored in the outer leaflet of neoplastic cell membranes may be shed into the extracellular tumor environment (25–27). This is supported by the observation that gangliosides with a similar migration pattern on TLC as those isolated from tumor supernatant were detected from the corresponding tumor tissue of the same patient. It was also observed that additional gangliosides, including monosialogangliosides, were detected in extracts from the tumor specimen that were not observed in the supernatant from the same tumor, raising the possibility that only select gangliosides are shed into the tumor environment. We investigated whether gangliosides isolated from the tumor tissue, like those from the corresponding supernatant, could inhibit induction of κB binding in T cells. The data presented in Figure 9 indicate similar inhibitory activity mediated by the tumor tissue–derived ganglioside.

Discussion

Tumors may escape immune rejection by inhibiting T-cell effector function. Defects in proliferation and cytokine production have been identified in both PBLs and tumor-infiltrating T lymphocytes (5, 6). The tumor environment and/or associated stroma may be responsible for the immune dysfunction through the production of a variety of immunosuppressive molecules (7, 28, 29). These products and others may facilitate evasion of the immune system by blocking intracellular signaling. For example, hydrogen peroxide derived from tumor-associated macrophages may be involved in depressing TCRζ levels in lymphocytes from tumor-bearing mice and cancer patients (30). Prostaglandins (PGE_2) and IL-

10, also present in the tumor environment, have been reported to inhibit κB binding activity in T cells (31–33). However, our studies demonstrate that the RCC-derived soluble product(s) that inhibits the activation of NFκB is distinct from these molecules based on the following findings: (a) the inhibitory product weighs less than 3 kDa and is therefore distinct from IL-10 and other higher-molecular-weight molecules; (b) the suppressive product is sensitive to neuraminidase but resistant to protease treatment; (c) the active material is water soluble and does not bind to a C18 column under acidic conditions; and (d) the inhibitory activity from tumor supernatants copurifies with gangliosides. Collectively, these findings suggest that gangliosides present in renal tumor supernatants are responsible for the inhibition of NFκB activation in T cells.

Gangliosides are structurally diverse acidic glycosphingolipids present in the outer leaflet of the plasma membrane of nearly all eukaryotic cells (25). However, malignant transformation can alter enzyme activation in ganglioside synthesis, resulting in aberrant expression of gangliosides by certain tumors. Incomplete ganglioside synthesis results in accumulation of precursor gangliosides G_{m3}, G_{m2}, G_{d3}, and G_{d2} in melanoma and G_{m2} and G_{d2} in neuroblastomas (34–36). Aberrant and elevated expression of gangliosides has been previously demonstrated on the surface of renal tumors (24, 37, 38). RCC biopsies and cell lines show an increase in G_{m2}, G_{m1}, and G_{d1a} types when compared with normal renal tissue. Several authors have correlated altered expression of select gangliosides to tumor progression and metastatic potential (39, 40). Ganglioside concentrations are highest in the tumor microenvironment, where they can be shed in the form of micelles or membrane vesicles (25). Our findings suggest that select gangliosides present on RCCs are shed as soluble products capable of inhibiting NFκB binding. Although both mono- and disialogangliosides were detected in RCC tissue, disialogangliosides predominated in the tumor supernatant. A recent study demonstrates that shed tumor cell gangliosides may exist as membrane vesicles, micelles, or monomers (25). However, it is noteworthy that the gangliosides isolated from RCC supernatants appear to exist as monomers (<3 kDa) and not in micelles. After the purification process, these gangliosides may aggregate into micelles as the concentration exceeds the critical micellar concentration.

Tumor-derived gangliosides have been previously reported to act as inhibitors of the host immune response (41–43) and appear to be involved in the immune dysfunction observed in patients with RCCs. Shed tumor–derived gangliosides have also been reported to inhibit murine allogeneic cellular response in vivo and human lymphocyte proliferative responses in vitro (44–46). Moreover, prevention of ganglioside shedding diminishes their immunosuppressive activity (27). This inhibitory action may involve multiple mechanisms, including defects in antigen presentation (47), cytotoxic effector function (48), and diminished production of select cytokines such as IL-1β, TNF-α, and IL-6 (46).

Our findings show that gangliosides isolated from the supernatant of RCC explants can inhibit IL-2 and IFN-γ production of peripheral blood T cells stimulated with PMA/ionomycin. Purified bovine brain gangliosides have recently been shown to inhibit the generation of specific mRNA transcripts encoding these cytokines at the level of transcription (41).

In the majority of, but not all, experiments, crude RCC supernatants and isolated gangliosides suppressed the nuclear accumulation of both the transactivating RelA/p50 dimer and the p50/p50 homodimer. However, experiments in which the nuclear expression of RelA/p50 was blocked, but that of p50/p50 was not, may suggest that the RelA/p50 dimer is more sensitive to ganglioside suppression than the homodimer of p50. It was also observed that suppression of NFκB activation with the commercial source of bovine brain–derived gangliosides was significant but not as complete as inhibition mediated by gangliosides derived from RCCs. One possible explanation is that because of structural differences, tumor-derived gangliosides are more immunosuppressive than gangliosides derived from normal bovine brain tissue, as reported previously (44).

The mechanisms by which gangliosides inhibit the activation of κB binding activity are not defined. Gangliosides are amphipathic and therefore capable of incorporating into cellular membranes, altering the function of host cells (25, 44). This may reflect the ability of gangliosides to modulate transmembrane signal transduction (25, 49–53). Because the stimulus-dependent degradation of IκBα was normal, the inhibitory mechanism did not target signaling events upstream of IκBα. Inhibitory gangliosides might act to prevent the nuclear translocation of Rel family proteins. Alternatively, ganglioside-induced suppression of NFκB activity may result from degradation of free Rel proteins by endogenous proteases. However, our recent experiments do not support the hypothesis that RelA is degraded. RelA immunoprecipitated from nuclear extracts of normal T cells was not degraded by either nuclear or cytoplasmic extracts from ganglioside-treated T cells. Additional experiments are under way to distinguish between Rel protein degradation and defective nuclear import.

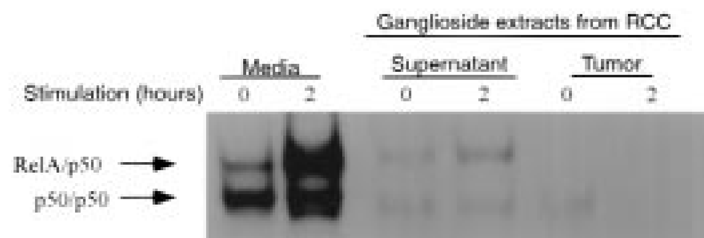

Figure 9

Gangliosides isolated from renal tumor tissue reproduce the suppression of NFκB activation observed with gangliosides derived from corresponding supernatant. Gangliosides were isolated from RCC tissue and RCC explant supernatant from the same patient's samples (*n* = 3). Their ability to inhibit κB binding activity of PMA/ionomycin–stimulated T cells was assessed by EMSA.

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