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Degradation of NF-κB in T Cells by Gangliosides Expressed on Renal Cell Carcinomas

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T cells from cancer patients are often functionally impaired, which imposes a barrier to effective immunotherapy. Most pronounced are the alterations characterizing tumor-infiltrating T cells, which in renal cell carcinomas includes defective NF-κB activation and a heightened sensitivity to apoptosis. Coculture experiments revealed that renal tumor cell lines induced a time-dependent decrease in RelA(p65) and p50 protein levels within both Jurkat T cells and peripheral blood T lymphocytes that coincided with the onset of apoptosis. The degradation of RelA/p50 is critical for SK-RC-45-induced apoptosis because overexpression of RelA in Jurkat cells protects against cell death. The loss of RelA/p50 coincided with a decrease in expression of the NF-κB regulated antiapoptotic protein Bcl-xl, at both the protein and mRNA level. The disappearance of RelA/p50 protein was mediated by a caspase-dependent pathway because pretreatment of T lymphocytes with a pan caspase inhibitor before coculture with SK-RC-45 blocked RelA and p50 degradation. SK-RC-45 gangliosides appear to mediate this degradative pathway, as blocking ganglioside synthesis in SK-RC-45 cells with the glucosylceramide synthase inhibitor, PPPP, protected T cells from tumor cell-induced RelA degradation and apoptosis. The ability of the Bcl-2 transgene to protect Jurkat cells from RelA degradation, caspase activation, and apoptosis implicates the mitochondria in these SK-RC-45 ganglioside-mediated effects. The Journal of Immunology, 2004, 172: 3480–3490.

Nuclear factor κB is a transcription factor involved in regulating proteins associated with cell proliferation, inflammation, host defense, and apoptosis (1–3). The NF-κB complex is made up of a variety of subunits, each containing a homologous region known as the Rel domain that binds to consensus sites of downstream target genes to initiate their transcription (4). The presence of a transcription activation domain is a feature that distinguishes RelA (p65), RelB, and c-Rel from the family’s smaller p50 and p52 subunits, which do not contain one. The most abundant forms of NF-κB transactivating complexes are the RelA/p50 heterodimers and the RelA/p65 homodimers (5). Before activation, these complexes are sequestered in the cytoplasm and are rendered inactive by inhibitory molecules belonging to the IκB family, with IκBα being the most predominant (6, 7). Following activation, the IκB becomes phosphorylated, ubiquitinated, and degraded by the 26 S proteasome (8), resulting in the release of NF-κB complexes and their nuclear translocation (6, 9). It was through the use of mutant IκBα constructs and RelA-negative mice that the important role of NF-κB in host immunity and antiapoptotic gene expression was established (8, 10, 11).

Defective activation of NF-κB has been reported in T cells from tumor-bearing mice and cancer patients (12–14). The deficiency stems from impaired nuclear localization of the RelA/p50 heterodimer, a problem that has been noted in both tumor-infiltrating lymphocytes (TIL)3 and peripheral blood T cells isolated from patients with renal cell carcinoma (RCC) (15–17). Coincident with impaired NF-κB is the demonstrated enhanced susceptibility of RCC patient T cells, particularly TILs, to apoptosis (18). Although ~10–15% of TIL are TUNEL-positive in situ, even those that are TUNEL-negative are sensitive to activation-induced cell death upon in vitro culture (17, 18). Interestingly, by performing in vitro experiments with tumor explant supernatants, it was determined that soluble products from RCCs can also induce the NF-κB defect and susceptibility to apoptosis in T cells from healthy controls (13, 18, 19). Uzzo et al. (19) reported that the inhibitory components of the tumor supernatants possessed several properties characteristic of a ganglioside, although the histological identity of the cell type(s) synthesizing the responsible glycosphingolipids remained uncharacterized. It was thus of interest to determine the cellular origin of the proapoptotic gangliosides, as well as the mechanism by which they might be mediating NF-κB inhibition and T cell dysfunction.

In this study we present evidence that the mechanism by which renal tumors inhibit NF-κB activation in peripheral blood T lymphocytes and the Jurkat cell line is by inducing the degradation of RelA and p50 proteins. The degradation of RelAp50 results in decreased DNA-binding activity and impaired expression of NF-κB regulated antiapoptotic proteins such as Bcl-xl. The loss of

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3 Abbreviations used in this paper: TIL, tumor-infiltrating lymphocyte; RCC, renal cell carcinoma; HA, hemagglutinin; SMC, smooth muscle cell; β-Gal, β-glucuronidase; PPPP, 1-phenyl-2-hexanoylamino-3-pyrrolidino-1-propanol.

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RelA expression is a critical event in tumor-induced apoptosis of T cells as demonstrated by the ability of RelA overexpression to protect Jurkat cells from decreased expression of Bcl-xL and apoptosis mediated by coculture with SK-RC-45. The mechanism of tumor-induced degradation of RelA/p50 involves a caspase-dependent pathway because inhibitors of caspase activity prevented disappearance of Rel proteins and abrogated T cell killing. Our studies also support the notion that tumor-derived gangliosides are important mediators of NF-κB inhibition and induction of apoptosis, because blocking ganglioside expression in RCC by pretreatment with the glucosylceramide synthase inhibitor, 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPPP), prevented both NF-κB inactivation and initiation of apoptosis by tumor.

Materials and Methods

Abs and reagents

Polyclonal goat IgG anti-RelA (C-20) and polyclonal rabbit IgG anti-p50 (H-119) Ab (Santa Cruz Biotechnology, Santa Cruz, CA) were used in Western blot analyses at a concentration of 2 μg/ml. Anti-Bcl-xL was a polyclonal rabbit IgG Ab (Santa Cruz Biotechnology). Monoclonal mouse IgG1 anti-actin (AC-15) from Novus Biologicals (Littleton, CO) was used at a dilution of 1/5000. Monoclonal mouse IgG anti procaspase 9 (Onco- gene Research Products, Boston, MA) and polyclonal rabbit anti-caspase-3 (BD PharMingen, San Diego, CA) specific for the pro-form (32 kDa) and active (12 and 17 kDa) fragments were used at 2 μg/ml. Secondary HRP-conjugated donkey anti-goat IgG was purchased from Santa Cruz Biotechnology. Sheep anti-mouse and donkey anti-rabbit secondary Abs, also HRP-conjugated, were purchased from Amersham (Arlington Heights, IL). Caspase inhibitor III and caspase 9 inhibitor II, both used at a concentration of 150 μM, were obtained from Calbiochem (La Jolla, CA). Active re- combinant caspase 3 enzyme was purchased from Calbiochem. The inhibitor of glucosylceramide synthase, PPPP, was purchased from Matreya (Pleasant Gap, PA). Preliminary studies suggested that 1.0 μM was most effective at blocking ganglioside synthesis and therefore this concentration was used in our experiments. PMA and ionomycin were purchased from Sigma-Aldrich (St. Louis, MO). G418 sulfate was purchased from Cellgro (Herndon, VA) and used at 1.0 μg/ml. Monoclonal anti-CD3 Ab (OKT3; Ortho Biotech, Raritan, NJ) and monoclonal anti-CD28 Ab (BD Immunocyto- metrics, San Jose, CA) were used for the stimulation of T lymphocytes. Human recombinant IL-2 (Proleukin; Chiron, Emeryville, CA) was used at 200 U/ml to maintain the viability of activated T cells.

Cell lines and tissue culture conditions

The Jurkat leukemic T cell line (American Type Culture Collection, Rock- ville, MD) was maintained in complete RPMI 1640 medium (BioWhit- take, Walkersville, MD) supplemented with 10% FCS (HyClone Labora- tories, Logan, UT), 2 mM L-glutamine, 50 μg/ml gentamicin, 100 mM MEM sodium pyruvate solution, and 10 mM MEM nonessential amino acid solution (Life Technologies, Grand Island, NY). HEK 293 cells (American Type Culture Collection) were maintained in complete DMEM (BioWhitaker) supplemented as previously described. The well-character- ized long-term RCC lines, SK-RC45, SK-RC-6, and SK-RC-54, were ob- tained courtesy of Dr. N. H. Bander (New York Presbyterian Hospital, Weil Medical College of Cornell University, Ithaca, NY). The SK-RC cell lines were allowed to reach confluence in 150-mm tissue culture dishes before use in coculture experiments with either Jurkat T cell or activated SMCs in the same 3:1 ratio as a negative control as were T cells cultured in medium alone. T cells and Jurkat cells devoid of adherent tumors were then assessed for NF-κB-binding activity, RelA/p50 protein levels, and for DNA breaks.

Treatment of Jurkat T cells with caspase inhibitors

Jurkat T cells were harvested and resuspended at 1 × 10^6 cells/ml in RPMI 1640 supplemented with 150 μM caspase inhibitor III (Calbiochem), a broad spectrum caspase inhibitor, or inhibitor II, specific for caspase 9. Cells were then allowed to incubate for 1 h before adding to SK-RC-45 cells that had been cultured for 5 days prior in 150-mm tissue culture dishes. Following an additional 48-h incubation, Jurkat cells were removed from the adherent SK-RC-45 monolayer as previously described. Whole cell lysates were prepared for Western blot analysis of RelA and p50.

EMSA

NF-κB activation was determined by stimulating Jurkat cells for 2 h with PMA (10 ng/ml) and ionomycin (0.750 μg/ml) before isolating nuclear extracts as previously described (19). Nuclear extracts were then prepared in binding reaction buffer as previously described (20). The binding reac- tions were performed with 10 μg of protein at room temperature for 30 min in a total volume of 25 μl. Oligonucleotide corresponding to the IL-2Rα promoter (5’-CAACGGCAGGGAATCTCCCTCTCCTT-3’) was obtained from Qiagen Operon (Alameda, CA) and used as a probe. Briefly, radiolabeled double-stranded oligonucleotides were prepared by annealing complementar- y strands and labeling with [α-32P]dCTP by nick translation according to the manufacturer’s recommended protocol (Roche, Mannheim, Germany). The samples were resolved on a 6% polyacrylamide gel with 0.25× TBE buffer (22.3 mM Tris, 22.2 mM boric acid, and 0.5 mM EDTA). The gels were dried and analyzed by autoradiography.

DNA fragmentation analysis by TUNEL assays

Cells were fixed in 1% paraformaldehyde, and were stained and analyzed for apoptosis using the APO-BrdU system (Phoenix Flow Systems, San Diego, CA). In brief, cells were labeled with 50 μl of DNA solution containing 10 μl of TdT reaction buffer. Cells were rinsed and resuspended in 0.1 ml of DNA solution containing fluorescein PRB-1 Ab. Propidium iodide/RNase A solution (0.5 ml) was added to each sample before incubation at room temperature for 30 min. Flow cytometric analysis was performed within 2 h of sample staining using FACSScan (BD Biosciences, Franklin Lakes, NJ) set to measure 1 × 10^6 events. Cells incubated in medium served as negative controls. HL60 promyelocytic leukemia cells induced with camptothecin were used as positive controls. The percentages of apoptotic T cells were obtained using quadrant analysis software (Lysis II; BD Biosciences).

Protein extracts and Western blot analysis

Cell pellets were resuspended in whole cell lysate buffer (1× PBS (calcium and magnesium free), 10 mM sodium pyrophosphate, 20 mM HEPES (pH 7.2), 1% Tween 20, 100 mM NaCl, 1 mM ZnCl2, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 100 μg/ml Pefabloc, and 0.1 μg/ml chymostatin, 100 μg/ml PMSF), for 30 min at 4°C. The lysate was centrifuged at 14,000 rpm for 10 min at 4°C, after which the supernatant was carefully transferred as to not disturb the pellet. Equivalent amounts of protein were mixed with an equal volume of 2× Laemmli buffer, boiled, and resolved on 10% SDS-PAGE gels. Following transfer to nitrocellulose membranes by electrobloodytting (Bio-Rad, Richmond, CA) as described (21), the blots were blocked with 5% nonfat dry milk in 1× TBST and subse- quently probed with the specific primary Abs previously described. The immunoreactive proteins were visualized using HRP-linked secondary Abs and ECL Western blotting kit (Amersham).
TUMOR-INDUCED DEGRADATION OF NF-κB

Plasmid constructs, transfections, and purification of recombinant RelA protein

A FLAG-tagged (N terminus) cDNA encoding human Bcl-2, a generous gift from Dr. G. Nunez (University of Michigan Medical School, Ann Arbor, MI), and an hemagglutinin (HA)-tagged (N terminus) cDNA encoding the RelA(p65) subunit of human NF-κB, a kind gift from Dr. J. DiDonato (Cleveland Clinic Foundation, Lerner Research Institute, Cleveland, OH), were subcloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA). Additionally, the HA-RelA cDNA was subcloned into the pcDNA4His/Max B (Invitrogen) vector containing an N-terminal 6x His tag. The resulting HA-RelA and Bcl-2 pcDNA3.1 plasmids were added to 5.0 × 10^6 Jurkat T cells at 10 μg each for transfection of stable cell lines with Lipofectamine (Invitrogen). Isolation of stably expressing clones was described. Following a 5-day selection with 250 μg/ml zeocin (Invitrogen), the cells were allowed to reach confluence. Thereafter they were washed using an NF-κB consensus site of the IL-2R-α promoter as a probe. B. Jurkat cells treated as described were harvested for TUNEL analysis after 48 h of coincubation to assess tumor-induced apoptosis. The average percentages of apoptosis obtained in Jurkat T cells were 4.93% (SD ± 2.18, n = 3) when cocultured with SMC and 58.84% (SD ± 13.54, n = 3) when cocultured with SK-RC-45.

In vitro cleavage assay for assessing degradation of recombinant RelA

Jurkat cells were cocultured with SK-RC-45 as described. Following a 48-h incubation, Jurkat cells were recovered and whole cell protein lysates were then prepared and quantified by the BCA method (Pierce, Rockford, IL). For the in vitro cleavage assay, 200 μg of Jurkat T cell lysates from cells either cultured in medium or cocultured with SK-RC-45, were combined with a 3% volume of the 1-mL recombinant RelA fraction. The reaction was incubated at 37°C for 2 h before being loaded onto a 10% SDS-PAGE for Western blot analysis with anti-RelA Ab.

Inhibition of glucosylceramide synthase in SK-RC-45 cells by PPPP

The glucosylceramide synthase inhibitor, PPPP, was added to the 50% confluent SK-RC-45 cell cultures to give the desired effective concentration of 1.0 μM PPPP (22). The final concentration of ethanol in the culture medium was ~1.0% (v/v). Following a 5-day exposure of the SK-RC-45 cells to PPPP, the drug was washed away, and the flask was replenished with fresh medium containing Jurkat cells or peripheral blood T cells. Following coculture, the nonadherent T cell targets were harvested and whole cell lysates were prepared for analysis of RelA and p50 by Western blot. The efficacy of the inhibitor PPPP was confirmed by high performance thin layer chromatography and orcinol detection of gangliosides isolated from both PPPP treated and nontreated SK-RC-45 cells as described (23).

Real-time PCR

Cytoplasmic total RNA was extracted from both null/pDNA3 and RelA/pDNA3 with Qiagen Midi RNA columns (catalogue no. 75142; Qiagen, Valencia, CA). The RNA was then DNase treated for 1 h at 37°C using RNase free DNase I from Ambion (Austin, TX). RT-PCR was performed using 5.0 μg total RNA and the TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) in the following reaction mixture: 0.25 mM each dNTPs, 2.5 mM MgCl₂, 20 U RNase inhibitor, 50 U Multiscribe reverse transcriptase, and 0.5 μM random hexamers, 1× reverse transcription reaction buffer. The RT-PCR was then cycled on a Perkin-Elmer/Cetus DNA thermal cycler (Norwalk, CT) at 65°C for 5 min, 15°C for 15 min, and 37°C for 45 min. Following RT-PCR, 2.0 μl of the cDNA was used in the subsequent PCR using SYBR green PCR core reagents (Applied Biosystems) in the following reaction mixture: 1× SYBR green

FIGURE 1. The RCC line, SK-RC-45, suppresses NF-κB activation and induces apoptosis in Jurkat T cells. A, Jurkat cells were cultured alone, with either SMCs or the SK-RC-45 cell line at a 3:1 ratio. Following a 48-h incubation, Jurkat cells were isolated and then stimulated for 2 h with 10 ng/ml PMA and 0.75 μg/ml ionomycin. Nuclear lysates prepared from the lymphocytes were subjected to EMSA analysis as described in Materials and Methods, using an NF-κB consensus site of the IL-2R-α promoter as a probe. B, Jurkat cells treated as described were harvested for TUNEL analysis after 48 h of coincubation to assess tumor-induced apoptosis. The average percentages of apoptosis obtained in Jurkat T cells were 4.93% (SD ± 2.18, n = 3) when cocultured with SMC and 58.84% (SD ± 13.54, n = 3) when cocultured with SK-RC-45.
PCR buffer, 2.5 mM MgCl₂, 1.0 mM dNTP/dUTP mixture, 0.25 U AmpliTaq Gold polymerase, 0.2 μM each primer. The following primers were used: Bcl-xL forward primer, 5'-AGCAAGCGCTGAG-3'; Bcl-xL reverse primer, 5'-GGTGGACCTCTTGTCTCAGTA-3'; β-glucuronidase (β-Gus) forward primer, 5'-TCTAGTGGTGATCGACATGCAG-3'; β-Gus reverse primer, 5'-ACTGGGTGCCTTTGTTGCGTGGACACGCC-3'. The samples were placed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems) set to the default parameters for thermal cycling conditions. When completed, the data were analyzed using the Dissociation Curve software available on the Applied Biosystems website (www.appliedbiosystems.com) to assure that nonspecific amplification had not occurred. The cycle threshold values obtained were then used to calculate the fold differences of Bcl-xL expression relative to the expression of the housekeeping gene, β-Gus.

**Results**

**Immunoinhibitory activity of SK-RC-45**

Both TILs and peripheral blood T cells isolated from RCC patients exhibit impaired NF-κB activation and increased sensitivity to apoptosis (16, 19). These defects can be reproduced in Jurkat T cells by coculturing the lymphocytes with the renal tumor line SK-RC-45 (Fig. 1). Prior supershift studies had shown that PMA/ionomycin stimulation of T cells results in two κB binding complexes; a slower migrating complex composed of RelA/p50 heterodimers and a faster migrating complex consisting of p50/p50 homodimers (20). EMSA analysis (Fig. 1A) demonstrated increased κB-binding activity of both the p50/p50 and transactivating RelA/p50 complexes following PMA/ionomycin stimulation. Coculturing Jurkat cells with SK-RC-45, however, almost completely abrogated stimulus-dependent κB-binding activity, whereas coincubation with SMC had no suppressive effect on NF-κB activation. The inhibitory effect of tumor cells on Jurkat cell NF-κB activation was paralleled by tumor-induced apoptosis of the lymphocytes (38%) as compared with SMCs, which did not mediate Jurkat cell apoptosis above the 5% background levels (Fig. 1B).

**SK-RC-45 induces degradation of NF-κB in Jurkat T cells**

We next assessed the possibility that SK-RC-45 might mediate NF-κB inhibition by reducing the expression levels of the RelA and p50 proteins in cocultured T cells. Indeed, Western blot analysis using Abs to RelA and p50, showed a time-dependent decrease in the levels of both proteins, which first became evident after a 48-h exposure to SK-RC-45 (Fig. 2A). The progressive decline in Jurkat cell RelA and p50 protein expression was accompanied by a coincident rise in the TUNEL positivity of those increasingly NF-κB-depleted cells (Fig. 2A). Two other long-term RCC cell lines, SK-RC-6 and SK-RC-54, similarly inhibited RelA protein levels in cocultured Jurkat cells that were accompanied by an induction of 43% and 34% of those lymphocytes, respectively, to undergo apoptosis (Fig. 2B). This suggests that the ability of SK-RC-45 to induce RelA degradation and apoptosis in T cells is not specific to that cell line, but is rather a general characteristic of RCCs.

**Overexpression of RelA in Jurkat cells protect from tumor-induced apoptosis**

The possibility that SK-RC-45-induced NF-κB degradation contributes to tumor-induced lymphocyte apoptosis led us to ask whether overexpressing RelA would protect Jurkat cells from SK-RC-45-mediated killing. Overexpression of RelA in Jurkat cells was confirmed by Western blot detection of the HA-tagged recombinant RelA, visible as a band of slightly higher m.w. compared with the endogenous form of RelA (Fig. 3A). Two Jurkat cell clones permanently transfected with the pcDNA3/HA-RelA construct were therefore compared with wild-type Jurkat cells for their susceptibility to SK-RC-45. In contrast to control Jurkat cells, 53% of which underwent apoptosis following a 48-h coincubation with SK-RC-45, only 13% and 11% of the RelA clone 1 and 2 transfectants, respectively, became TUNEL-positive following similar treatment (Fig. 3B). Jurkat cells expressing the vector alone were not protected from apoptosis. These results suggest that tumor-mediated inhibition of NF-κB via degradation of the RelA and p50 proteins plays a significant role in rendering T cells sensitive to tumor-derived apoptogenic products.

**Tumor-induced loss of RelA/p50 coincides with impaired expression of NF-κB-dependent genes**

Given that RelA/p50 initiates transcription of antiapoptotic genes and that coculture with SK-RC-45 inhibits NF-κB activation, it was of interest to know whether exposure to tumor caused a decrease in expression of NF-κB-dependent antiapoptotic genes, such as Bcl-xL. Wild-type Jurkat cells and the RelA-overexpressing cells were cultured in the presence or absence of SK-RC-45, before assessing their expression of Bcl-xL protein and mRNA. Coculture...
with tumor caused a significant decrease in Bcl-xL protein levels when compared with Jurkat cells cultured in medium alone (Fig. 4A, compare lanes 1 and 3 and Fig. 4B). Predictably, the RelA-overexpressing clone elaborated enhanced levels of Bcl-xL protein as compared with the wild-type Jurkat cells, (Fig. 4A, compare lanes 1 and 2 and Fig. 4B), which interestingly were not significantly diminished...
The tumor-induced inhibition of NF-κB appreciably affected by exposure to tumor. These results are not unique to Bcl-xL because coculturing Jurkat cells with SK-RC-45 also inhibited expression of the Bcl-2 protein (23).

**The mechanism of SK-RC-45-mediated degradation of NF-κB involves a caspase-dependent pathway**

A tumor-induced decrease in Rel protein levels in Jurkat cells may stem from an SK-RC-45-mediated proteolytic event. To address this issue, we compared the ability of lysates from tumor-exposed and unexposed Jurkat cells to cleave a recombinant, HA-tagged RelA protein in vitro. When control Jurkat cell lysates, to which recombinant RelA had been added, were subjected to Western blot analysis, anti-RelA Abs detected both the endogenous protein, and the slow-running transgenic form, rendered slightly larger by virtue of its HA tag (Fig. 5A). When an equivalent amount of recombinant RelA was incubated with the lysates made from tumor-exposed Jurkat cells, the HA-tagged protein was reduced to a fragment of its HA tag (Fig. 5A). These results suggest that a Jurkat cell protease activated during coculture with SK-RC-45 plays a role in tumor-mediated inhibition of NF-κB activation.

Involvement of caspases in Rel protein decay was confirmed by the data presented in Fig. 6A. As compared with the basal expression levels of RelA and p50 detected in lysates from untreated Jurkat cells, Western analysis revealed that those proteins were diminished by ~70% and 95%, respectively, in extracts made from Jurkat cells cocultured with SK-RC-45. This decay of RelA and p50 was completely abrogated, however, if the coincubation of Jurkat cells with tumor took place in the presence of 150 μM pan caspase inhibitor III. When Jurkat cells were treated with the caspase 9 inhibitor during coincubation with tumor cells, SK-RC-45-induced degradation of RelA/p50 was completely blocked. The ability of the pan caspase inhibitor III and caspase 9 inhibitor to protect Jurkat cells from both Rel protein decay (Fig. 6, A and C) and apoptosis (Fig. 6, B and D), underscores the integral roles that mitochondria and caspase 9 play in both phenomena (Fig. 6).

To further assess the requirement for mitochondrionally activated caspases in tumor-induced apoptosis and Rel protein degradation, we determined the ability of SK-RC-45 to mediate RelA and p50 decay in Jurkat cells overexpressing Bcl-2, an antia apoptotic molecule that protects mitochondria against cytochrome c release and subsequent caspase 9 activation (24, 25). Compared with wild-type Jurkat cells cultured in medium, which showed no evidence of caspase activation, active fragments of caspase 3 (12/17 kDa) were detected in lysates from the SK-RC-45 cocultured T cells, as was a decrease in their expression level of procaspase 9. Activation of caspases 9 and 3 that led to Jurkat cell apoptosis (data not shown) was completely abrogated in Bcl-2 overexpressing Jurkat cells (Fig. 7A), and correlated with the capacity of the transgene to also prevent tumor-induced apoptosis (26).

Degradation of RelA and p50 induced by SK-RC-45 was also abrogated in the Bcl-2 transfectants. Lysates from wild-type Jurkat cells cocultured with SK-RC-45 degraded both endogenous and exogenously added recombinant RelA in vitro, although extracts from Bcl-2 cells coincubated with tumor cells under the same conditions were incapable of degrading those molecules (Fig. 7, B and C). Wild-type and Bcl-2 transfected Jurkat cells cultured in medium alone served as negative controls. These findings suggest that caspase activation is a necessary prerequisite for NF-κB degradation.

Caspases themselves did not play a direct role in Rel protein decay. RelA degradation was not blocked when the pan caspase inhibitor III and recombinant RelA were added directly to lysates made from Jurkat cells that had been cocultured with SK-RC-45. Although the pan caspase inhibitor prevented commercial caspase 3 from degrading DFF-45, it had no detectable protective effect on extract-mediated cleavage of recombinant RelA (our unpublished observations). Similar results were obtained with the caspase 9 inhibitor. These findings suggest that caspases activated within Jurkat cells during coculture with tumor, themselves activate downstream, noncaspase proteases, which directly mediate the observed degradation of RelA and p50.

**SK-RC-45 derived gangliosides suppress NF-κB in T cells**

In this series we asked whether gangliosides expressed by SK-RC-45 contributed to the Rel protein degradation observed within cocultured Jurkat cells. Jurkat T cells were coincubated with SK-RC-45 monolayers that had been pretreated or not for 5 days with 1 μM of the glucosylceramide synthase inhibitor PPPP, a concentration that we as well as others have found to consistently reduce by exposure to tumor (Fig. 4, compare lanes 2 and 4). Similar findings were observed when Bcl-xL mRNA levels were examined in the same experiments using quantitative PCR. As seen in Fig. 4C there was decreased Bcl-xL mRNA in Jurkat cells incubated with SK-RC-45 compared with Jurkat cells (null-pcDNA3) cultured in medium alone. Bcl-xL mRNA level was elevated in the RelA clone that was not appreciably affected by exposure to tumor. These findings suggest that the tumor-induced inhibition of NF-κB transcriptional activity in Jurkat cells results in the reduced expression of Bcl-xL mRNA and protein. These results are not unique to Bcl-xL because coculturing Jurkat cells with SK-RC-45 also inhibited expression of the Bcl-2 protein (23).

**FIGURE 5.** Cytoplasmic lysates made from SK-RC-45 cocultured Jurkat cells mediate proteolysis of recombinant RelA (rRelA) in vitro. A, Jurkat cells were cocultured or not with SK-RC-45 at a 3:1 tumor to target cell ratio for 48 h. B, As a control Jurkat cells were also cocultured with SMCs under the same conditions. Cytoplasmic lysates made from the isolated lymphocytes were assessed for their ability to degrade RelA by coincubating 200 μg of the extracts with a 3% v/v aliquot of recombinant HA-tagged RelA at 37°C for 2 h. Equal volumes of the reaction mixtures were run on an SDS-PAGE gel, which was subsequently blotted and analyzed by Western blot analysis using an Ab to RelA. The data presented were representative of three separate experiments.
ganglioside expression by 70% (23, 27). Lysates prepared from Jurkat cells following coincubation with both PPPP treated and untreated tumor were analyzed for p50 and RelA expression. The lysates from Jurkat cells exposed to ganglioside-expressing SK-RC-45 had 10% of the RelA and p50 protein levels seen in control Jurkat cells cultured in medium alone (Fig. 8A). This SK-RC-45-mediated depletion of Rel proteins was associated with the ability of the tumor line to induce 52% of the Jurkat lymphocytes to undergo apoptosis (Fig. 8B). These findings were in contrast to those obtained with the PPPP-pretreated SK-RC-45, which not only induced minimal levels of apoptosis (<10%), but also did not induce RelA or p50 degradation in the Jurkat cells (Fig. 8A).

The potential, physiological relevance of ganglioside-induced Rel protein degradation was confirmed by repeating the experiment using normal peripheral blood T cells. As seen with Jurkat cells, ganglioside-synthesizing SK-RC-45 significantly reduced RelA expression within peripheral blood T cells (Fig. 9, A and B). Parallel to this finding was the ability of SK-RC-45 to induce ~22% of those lymphocytes to undergo apoptosis (Fig. 9C). The fact that PPPP pretreatment of SK-RC-45 similarly inhibits tumor-induced RelA degradation and minimizes apoptosis in normal, activated T cells, suggests that ganglioside expression is an immune escape mechanism of tumors.

**Discussion**

Our study illustrates a potential mechanism by which renal cell carcinoma lines induce apoptosis in T cells. Gangliosides expressed by SK-RC-45 stimulate a sequence of events leading to the inhibition of NF-κB (1). Collectively our data suggest that gangliosides expressed by RCC lines can activate the mitochondrial pathway of apoptosis by initiation of the caspase cascade via caspase 9. Activated caspases in turn stimulates a noncaspase protease to degrade the NF-κB transactivating complex, RelA/p50. The loss of this NF-κB complex further promotes apoptosis through decreased expression of select antiapoptotic genes, including Bcl-xL and Bcl-2, that are controlled by NF-κB. Overexpression of Bcl-2 and RelA in Jurkat cells prevented ganglioside-mediated RelA/p50 degradation, and minimized tumor-induced lymphocyte apoptosis, suggesting a critical role for NF-κB in protecting T cells from the tumor microenvironment.

Gangliosides are structurally diverse acidic glycosphingolipids present in the outer leaflet of the plasma membrane of cells (28) that are involved cell differentiation, growth, cell adhesion (29, 30) and can also serve as receptors for microbes and their toxins (31). Tumors often exhibit augmented synthesis of select gangliosides, which are shed into the tumor microenvironment (32, 33). For example, malignant melanomas and neuroblastomas overexpress...
GD3, GD2, and GM2, (34) while increased expression of GD1a, GM1, and GM2 has been noted in renal cell carcinomas (35). Tumor gangliosides are now thought to participate in tumor formation and progression (36). Indeed, the administration of tumor-derived GM1b to mice has been found to promote malignant growth (37), whereas enhanced RCC expression of several disialogangliosides has been correlated with the increased metastatic potential of renal tumors (38). Gangliosides have these effects perhaps in part because of their ability to inhibit host immunity. This includes their ability to suppress T cell proliferation, dendritic cell differentiation and Th1 cytokine production in vitro (37, 39–44).

Gangliosides isolated from supernatants of RCC explants suppress NF-κB activation in peripheral blood T cells (19). In this study we demonstrate that gangliosides expressed by the renal cell carcinoma lines contribute to tumor-induced NF-κB suppression and apoptosis of T cells. Inhibiting SK-RC-45 glycosphingolipid expression using PPPP neutralized both tumor-induced RelA degradation and apoptosis in T cells (Figs. 8 and 9). Given that PPPP inhibits most glycosphingolipids, the evidence that it is gangliosides expressed by the tumor that are responsible for the suppression of NF-κB is supported by two observations. First, the renal line SK-RC-54 that expresses GM2 can induce apoptosis of T cells that can be partially blocked (›50%) by the addition of an Ab to GM2. The isotype control Ab, however, had no effect (our unpublished observations). Second, we have found that a mixture of bovine brain derived gangliosides (GM1, GD1a, and GD3), when cocultured with T cells, results in the loss of RelA protein expression and cell death (C. Ng, P. Rayman, T. Bloom, and J. Finke, manuscript in preparation). The exact identity of the gangliosides expressed by SK-RC-45 that are responsible for suppressing NF-κB activation and inducing apoptosis are not yet known. However, bovine brain derived gangliosides GM1, GD1a, and GD3 are reported to inhibit κB-binding activity in both T cells and hepatocytes (19, 39, 45). Given that renal tumors show increased expression of GM1 and GD1a, whereas melanoma overexpressed GD3, suggest that these tumor-derived gangliosides may promote NF-κB suppression. The fact that GM3 does not inhibit NF-κB-binding activity (19, 39, 45) indicates that only select gangliosides are involved in this process. This notion is supported by recent ganglioside fractionation studies showing that only some ganglioside peaks suppressed NF-κB and induced apoptosis (P. Rayman, R. George, and J. Finke, unpublished observations). Given that gangliosides are heterogeneous in their ability to suppress immune cells, the composition of gangliosides expressed by a given tumor may dictate the type and severity of immune alterations that may occur.

Additional studies presented here examined how SK-RC-45-derived gangliosides impair NF-κB activation and sensitize T cells to apoptosis. The mechanism by which SK-RC-45 inhibits NF-κB activation does not involve interference with normal IκBα degradation or sequestration of Rel-containing complexes within the...
cytoplasm. Thus, the early events in the pathway of NF-κB signaling that occur before IκBα degradation, are not affected by tumor derived gangliosides. We instead find that a sustained loss of NF-κB activity in T cells is the result of an active degradation of RelA(p65)/p50 proteins, stimulated by exposure of these cells to gangliosides expressed by renal carcinoma.

The mechanism used by SK-RC-45 gangliosides to inhibit NF-κB activation in T cells apparently differs from that used by

FIGURE 9. Pretreatment of SK-RC-45 with the glucosylceramide synthase inhibitor PPPP abrogates tumor-induced apoptosis of activated, peripheral blood T cells, and Rel protein degradation. A, Anti-CD3/CD28-activated peripheral blood T cells (PBL-T) were cocultured for 48 h with SK-RC-45 tumor cells, which were pretreated or not for 5 days with PPPP. Whole cell lysates subsequently prepared from the cocultured cells were subjected to Western blot analysis using Abs to RelA, p50 and actin, to assess the ability of the ganglioside synthesis inhibitor to abrogate tumor-induced degradation of the NF-κB family proteins. Mean pixel numbers (B) obtained by densitometry of the Western blot presented in A. C, Peripheral blood T cells cocultured with the SK-RC-45 tumor cells (A) were subjected to TUNEL analysis, to assess the ability of the glucosylceramide synthase inhibitor to abrogate tumor-induced apoptosis of the lymphocytes. The data presented were representative of three separate experiments.
GD3 to mediate that effect in hepatocytes (45). Although GD3 also suppresses hepatocyte NF-κB activation by a mechanism that allows for normal IκBα degradation, RelA and p50 proteolytic decay are not observed (45). In hepatocytes, GD3 blocks NF-κB activation by inhibiting the nuclear import of RelA/p50 (45). Interestingly, GD3 treatment of T lymphocytes inhibits NF-κB activation in those cells not by the mechanism defined in GD3-treated hepatocytes, but rather by the proteolytic pathway described here for SK-RC-45 ganglioside-exposed T cells (C. Moon, T. Bloom, A. Richmond, P. Rayman, and J. Finke, unpublished observations). These findings suggest that, perhaps generally, ganglioside-mediated inhibition of NF-κB activation in T cells results from degradation of RelA/p50 and not from inhibiting NF-κB nuclear import. Furthermore, because RCCs express considerably less GD3 than normal kidney (35), that ganglioside is unlikely to mediate the immunosuppressive effects induced by RCC.

Our current findings suggest that the degradation of NF-κB is dependent upon caspase activation following T cell exposure to RCC-derived gangliosides (Fig. 6). This observation prompted us to explore the possibility that RelA and p50 are substrates for direct proteolysis by an active caspase. However, the addition of various caspase inhibitors to lysates from tumor-exposed Jurkat cells was ineffective at preventing degradation of RelA. These findings differ from those published previously that suggested that RelA is degraded by caspase 3 in cells undergoing apoptosis (46, 47). Indeed, these reports cited the presence of consensus caspase 3 cleavage sites, however, the region in which RelA was said to be cleaved varies greatly. Additional studies suggest that RelA is a substrate for both caspases 3 and 6, with cleavage resulting in an endogenously formed and transcriptionally inactive dominant-negative NF-κB mutant (48). Given the controversy surrounding the identities and Rel cleavage sites of the responsible caspase(s), we believe that cleavage of RelA/p50 is a cell system and stimulus dependent mechanism. Although caspase activation is requisite for RelA/p50 degradation, our findings suggest that caspases are not directly degrading RelA/p50 in T cells. Others have actually proposed a completely caspase-independent mechanism of NF-κB suppression involving the proto-oncogene, c-myc (49).

Our study also indicates a central role for the mitochondrion in the activation of caspases, NF-κB degradation, and sensitization of T cells to apoptosis by RCC-derived gangliosides. Mitochondria amplify receptor-dependent signals in those cell types that do not generate adequate DISC formation following binding of death ligands, as in Jurkat cells and T lymphocytes through the activation of Bid (50, 51). Activated tBid binds to and induces the oligomerization of Bak and Bax in the mitochondrial membrane, leading to cytochrome c release and activation of caspase 9 (52–55). Mitochondria are also at the apex of a second apoptotic pathway induced by environmental stresses such as ionizing radiation and cytotoxic drugs (56–58). The conclusion that mitochondria participate in SK-RC-45-mediated NF-κB degradation and apoptosis in T cells derives from our finding that Bcl-2 overexpression in Jurkat cells abrogates both effects. Bcl-2 likely functions by stabilizing mitochondrial membranes and preventing cytochrome c release caused by apoptogenic stimuli (24, 59–61), including renal tumors. Data are also accumulating that indicate select gangliosides, such as GD3 and GM1, can induce apoptosis by damaging the mitochondrion of hepatocytes (45). We have found that gangliosides isolated from RCC supernatants induce mitochondrial permeability transition and cytochrome c release from T cells which in turn activates caspases (T. Das, P. Rayman, G. Sa, and J. Finke, manuscript in preparation).

In addition to inhibiting NF-κB activation, SK-RC-45 cells have been reported to stimulate the decay of Bcl-2 in T cells (23). This phenomenon is also ganglioside-dependent, based on the inability of PPPP pretreated tumors to induce the effect (23). In this study we show that the degradation of RelA/p50 in Jurkat cells also coincides with decreased expression of another antiapoptotic protein, Bcl-xL. Given that the transcription of Bcl-xL is tightly regulated by NF-κB, it is likely that the loss of RelA/p50 is responsible for the decrease in Bcl-xL expression in Jurkat cells cocultured with SK-RC-45. This idea is supported by the observation that coculturing Jurkat cells with tumor resulted in decreased Bcl-xL mRNA expression (Fig. 4). Thus, tumor-derived gangliosides may promote apoptosis by not only indirectly inducing the degradation of RelA/p50 but also by inhibiting expression of the antiapoptotic proteins, Bcl-xL and Bcl-2.

NF-κB is a ubiquitously expressed transcription factor controlling genes related to cell regulation, proliferation, and survival (62–64). The role of NF-κB in T cell survival was confirmed by Kolenko et al. (21), who demonstrated that inhibition of NF-κB with the SNS50 peptide led to an apoptotic response. The functional importance of NF-κB to T cell survival was further established in studies demonstrating a mutant IκBα repressor, constitutively inhibiting NF-κB, could sensitize Jurkat cells to TNF-α-induced apoptosis (10). The ability of tumor-derived gangliosides to mediate both NF-κB degradation and T cell apoptosis, on the one hand, and the finding that the RelA transgene can protect Jurkat cells from SK-RC-45 induced death, on the other, suggests that NF-κB inhibition may be an immune escape mechanism used by RCC. Our current research would therefore imply that deployment of inhibitors targeting ganglioside synthesis, thus protecting lymphocytes from NF-κB degradation, might be effective approaches to enhancing T cell responsiveness in a tumor-bearing host.

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