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Renal Cell Carcinoma Tumors Induce T Cell Apoptosis through Receptor-Dependent and Receptor-Independent Pathways

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Tumors can promote their own progressive growth by inducing T cell apoptosis. Though previous studies suggested that tumor-mediated T cell killing is receptor dependent, we recently showed that tumor gangliosides also participate, a notion consistent with reports indicating that, in some cell types, gangliosides can activate the intrinsic apoptotic pathway by stimulating reactive oxygen species production, cytochrome c release, and caspase-9 activation. In this study, we used normal peripheral blood T cells, as well as caspase-8-, caspase-9-, and Fas-associated death domain protein-deficient Jurkat cells, to assess whether the death ligands and gangliosides expressed by the renal cell carcinoma (RCC) cell line SK-RC-45 can independently stimulate T cell apoptosis as a mechanism of immune escape. Anti-FasL Abs and the glycosylceramide synthase inhibitor 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPPP) each partially inhibited the ability of SK-RC-45 to kill cocultured activated T cells; together, as purified molecules, RCC gangliosides and rFasL induced a more extensive mitochondrial permeability transition and greater levels of apoptosis than either agent alone, equivalent to that induced by the FasL- and ganglioside-expressing RCC line itself. rFasL-mediated apoptosis was completely inhibited in caspase-8- and Fas-associated death domain protein-negative Jurkat cells, though apoptosis induced by purified gangliosides remained intact, findings that correlate with the observed partial inhibition of SK-RC-45-induced apoptosis in the Jurkat lines with defective death receptor signaling. Western blot analysis performed on lysates made from wild-type and mutant Jurkat cells cocultured with SK-RC-45 revealed caspase activation patterns and other biochemical correlates which additionally supported the concept that tumor-associated gangliosides and FasL independently activate the caspase cascade in T cells through the intrinsic and extrinsic pathways, respectively. The Journal of Immunology, 2008, 180: 4687–4696.

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Abbreviations used in this paper: RCC, renal cell carcinoma; PPPP, 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol; FADD, Fas-associated death domain protein; MPT, mitochondrial permeability transition; NAT, tumor-adjacent normal tissue; NKE, normal human kidney epithelial; DAPI, 4',6'-diamidino-2-phenylindole; HP TLC, high performance thin layer chromatography.
with enhanced tumor-induced killing of T cells (29); and tumor cells preincubated with the glycosylceramide synthase inhibitor 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPPP) were inhibited by 50% in their capacity to kill activated T cells (30). However, the exact role of RCC-derived gangliosides in tumor-induced T cell killing remains unclear. Previous studies from our laboratory indicated that ganglioside-producing RCC lines induced apoptosis of cocultured activated T cells by a mechanism that involved mitochondrial cytochrome c release and caspase-9 activation, suggesting the possibility of a direct, receptor-independent effect of gangliosides on mitochondria as has been suggested previously for other cell types (30). This result, however, is also consistent with a known role for mitochondria in merely amplifying apoptosis following activation of caspase-8 and the cleavage of Bid (31). Indeed, because many of these studies were performed using the Jurkat line, “type II” cells which absolutely require mitochondrial amplification of death receptor-induced signals to effectively initiate the apoptotic pathway (32), it has been difficult to address whether specific tumor-derived molecules can mediate apoptosis of normal, peripheral blood T cells by independently activating the extrinsic or intrinsic apoptotic pathways, or whether receptor-dependent and independent signals must act collectively through both pathways simultaneously or successively to kill the lymphocytes.

In the studies reported here, we used pharmacological inhibitors of both caspases-8 and -9, as well as Jurkat cell lines deficient in caspase-8, caspase-9, or Fas-associated death domain protein (FADD) to assess the involvement of receptor-dependent and -independent pathways in RCC-mediated T cell apoptosis and/or pro-apoptotic events. SK-RC-45 is an RCC cell line that overexpresses multiple gangliosides (29) and FasL (7), affording us the opportunity to assess whether RCC-derived products can independently and simultaneously mediate T cell apoptosis through the receptor-dependent (FasL) and -independent (gangliosides) pathways. Our results indicate that the RCC line SK-RC-45 synthesizes tumor-associated products that can initiate proapoptotic events through both the extrinsic and intrinsic apoptotic pathways, with FasL first activating caspase-8, and tumor gangliosides capable of directly inducing the mitochondrial permeability transition (MPT) and activating caspase-9.

### Materials and Methods

#### Reagents

An agonistic anti-Fas Ab was purchased from Upstate Biotechnology, and was used at a concentration of 0.5 μg/ml to induce apoptosis of Jurkat T cells through the FasR. This same Ab was used at 1.0 μg/ml for determining Fas expression levels by Western analysis for cell surface levels of FasR, binding of a primary, nonagonistic anti-Fas Ab (BD Pharmingen) was detected using a secondary, anti-mouse FITC-conjugated rabbit anti-mouse Ab (BD Pharmingen). A blocking anti-FasL Ab (Ab-1; Oncogene Research Products) was used at 4 μg/ml to antagonize FasL. A blocking anti-CD3 Ab (OKT3; Ortho Biotech) and monoclonal anti-CD28 Ab (BD Immunocytometry Systems) were used for the stimulation of T lymphocytes. HPTLC analysis of the tumor-derived gangliosides was performed using a goat polyclonal IgG used at 2 μg/ml (Santa Cruz Biotechnology), and murine mAbs to procaspases-8 and -9 were obtained from Oncogene Research Products and were used at 2 μg/ml. Polyclonal rabbit anti-BID Ab was also purchased from BD Pharmingen, and was used at a 1:5000 dilution. FADD expression was assessed with an anti-FADD murine monoclonal Ab (BD Pharmingen), and was used at 1 μg/ml. Monoclonal anti-CD3 Ab (OKT3; Ortho Biotech) and monoclonal anti-CD28 Ab (BD Immunocytometry Systems) were used for the stimulation of T lymphocytes. Pan-caspase inhibitor III, as well as inhibitors to caspases-8 and -9, were obtained from Calbiochem. Human IL-2 (Aldesleukin (Proleukin); Immunocytometry Systems) were used for the stimulation of primary T cells. The ganglioside synthesis inhibitor PPPP was purchased from Matreya. Etoposide (VP-16-213) was purchased from Sigma-Aldrich, and was used at 10 μg/ml overnight for inducing apoptosis of Jurkat cells. The Ab used to measure cytochrome c release was a mouse anti-human Ig purchased from BD Pharmingen. A murine anti-actin Ab (Santa Cruz Biotechnology) was used at 2 μg/ml in Western analysis to verify equal loading of protein.

#### Cell culture

Peripheral blood T cells were obtained from healthy donors with informed consent, and were isolated by negative selection and activated with anti-CD3/CD28 as described previously (30). The Jurkat leukemia T cell line was purchased from American Type Culture Collection and was maintained in complete medium (RPMI 1640; BioWhittaker) as described previously (30). Well-characterized caspase-8-negative and FADD-negative Jurkat cell lines, generated by treating Fas-sensitive cells with the franking shift mutagen ICR191 followed by selection with anti-Fas, were gifts from J. Blenis (Harvard Medical School, Cambridge, MA) (33). Jurkat cells expressing dominant/negative forms of FADD or caspase-8 were generated using the appropriate constructs, as described previously (34). Well-characterized, long-term RCC lines (SK-RC-45, a line expressing FasL and tumor-associated gangliosides (29) and SK-RC-48) (35) were obtained from Dr. N. Bander (New York Hospital, Cornell University Medical College, New York, NY). The RCC cell lines were maintained in complete RPMI 1640 medium at 37°C with 5% CO₂, and were allowed to reach confluence in 150-mm dishes before use in coculture experiments with Jurkat cell or normal peripheral blood T cell populations. RCC1 and RCC2 were short-term RCC lines generated from two individual explanted RCC tumors, and grown in culture for several passages. Excised renal tumors also contained normal adjacent tissue, which was isolated and individually processed to provide short-term cell lines of control kidney epithelium (tumor-adjacent normal tissue (NAT) that could be used as negative controls for some studies. A normal human kidney epithelial (NKE) cell line was obtained from the American Type Culture Collection, and was used in coculture experiments to control for the long-term RCC tumor lines.

#### Induction of apoptosis in primary T cells and Jurkat cell lines

To obtain cells specifically induced to apoptosis through the Fas or mitochondrial pathways, Jurkat cells were incubated for 24 or 48 h with either agonistic anti-Fas Ab (0.5 μg/ml), or etoposide (10 μg/ml), respectively. For some experiments, treatment with FasL was performed using potently bioactive FasL-expressing vesicles, which are blebbled off or secreted from packaging cell lines transfected with FasL-encoding retroviral vectors, as described previously (36, 37). The virus-containing supernatants are not infectious for Jurkat cells due to the ectopic nature of the packaging cell line, and hence the selective effects of the supernatants were independent of transduced gene expression (36). The ability of tumor cells to induce T cell apoptosis was assessed by adding 2.5 × 10⁶ primary T cells or Jurkat cells in fresh medium to confluent plates of 8 × 10⁴ SK-RC-45 or SK-RC-48 cells containing an equal volume of the 7-day tumor-conditioned medium, and letting the incubation proceed for 48–72 h. Following coculture, the nonadherent Jurkat cells were detached from the tumor cell monolayers by gentle washing, and then processed for either TUNEL, nuclear blebbing (4,6’-diamidino-2-phenylindole (DAPI) staining) or Western analysis, as described previously (38). Coincubation of T lymphocytes with normal kidney epithelial cells served as a negative control.

#### FasL mRNA analysis by RT-PCR

RNA was isolated from normal adjacent tissue, three long-term RCC cell lines, and three RCC tumors immediately after surgery by the guanidine isothiocyanate/cesium chloride method, followed by ethanol precipitation and storage at −70°C. The RT-PCR were performed with the sense and antisense primers for FasL used previously and as described before (7). The PCR products were separated by agarose gel electrophoresis and visualized by Southern hybridization analysis using the radiolabeled oligonucleotide probe previously described (7).

#### Ganglioside isolation and high performance thin layer chromatography (HPTLC) analysis

Gangliosides were isolated from SK-RC-45 tumor cells as described before (30), quantified using the lipid-bound sialic acid assay as outlined previously, (29), and then used at a concentration of 25 μg/ml to treat peripheral blood T cells and Jurkat cells. HPTLC analysis of the tumor-derived gangliosides was also performed as described previously to identify the gangliosides overexpressed by the fresh RCC isolates (29).

#### Immunocytochemistry of Fas expression by Jurkat cell lines

Jurkat cells were stained with Abs to FasR. Anti-CD95 (BD Biosciences) was purchased as a FITC conjugate, and was matched with an isotype control. Cells were washed in HBSS (BioWhittaker), and individual samples were divided
for assessing autofluorescence or staining with anti-CD95. Cells were then fixed in 1% paraformaldehyde and analyzed by flow cytometry (FACSCalibur; BD Biosciences). Live gating of the forward and orthogonal scatter channels was used to exclude debris and to selectively acquire Jurkat cell events. Individual fluorescence data were determined through the use of acquisition and quadrant analysis software (CellQuest; BD Biosciences). Percentages of cells expressing CD95 were calculated, and statistical analyses were performed using the Kruskal-Wallis and Wilcoxon’s rank sum tests.

Analysis of DNA fragmentation by TUNEL assay

Cells were fixed in 1% paraformaldehyde, and were stained and analyzed for apoptosis using the APO-BrdU kit system (Phoenix Flow Systems; Ref. 39). Briefly, Jurkat 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 a solution containing fluorescein PRB-1 Ab. A 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 a FACScan (BD Biosciences) set to measure 10,000 events. Uninduced T cells served as negative controls. The percentages of apoptotic T cells were obtained using quadrant analysis software (LYSIS II; BD Biosciences).

Cell lysates and analysis of protein by Western blotting

Cell pellets were resuspended in lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT) containing protease inhibitors (5 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM PMSF, 100 μg/ml pefabloc, and 100 μg/ml chymostatin). The cell pellet was resuspended in 100 μl of mitochondrial isolation buffer (20 mM HEPES-KOH, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, and 250 mM sucrose, containing protease inhibitors (5 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM PMSF, 100 μg/ml pefabloc, and 100 μg/ml chymostatin). The cell pellet was resuspended in 100 μl of mitochondrion isolation buffer, and following 20 min on ice, was homogenized with a Dounce homogenizer. The homogenate was centrifuged at 750 × g for 20 min, and the supernatant, which contained released cytochrome c, assessed for protein concentration. Equivalent amounts of protein were mixed with an equal volume of 2× Laemmli buffer, boiled, and resolved on 12% SDS-PAGE gels.

Assessment of mitochondrial membrane potential

Mitochondrial membrane potentials were measured by DiOC6 staining as described previously (41). Briefly, 3 × 10⁶ control Jurkat cells, caspase-8-negative Jurkat cells, FADD-negative Jurkat cells, caspase-9 ΔN Jurkat cells, or activated peripheral blood T cells were cocultured with SK-RC-45 or incubated with RCC-derived gangliosides or rFasL alone or in combination for 18 h, before isolating the lymphocytes and incubating them for an additional 15 min at 37°C in 500 μl of 40 nM/L DiOC₆. The cells were then analyzed immediately in FACS analysis with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Statistical analysis

The Student t test (paired two samples for mean or two samples using equal variances) was used to determine p using Microsoft Excel Software (version 2003). SEM was calculated from SD using Microsoft Excel software.
Results

Apoptogenic RCC tumor cells synthesize FasL and gangliosides, which independently induce T cell apoptosis

When normal resting, peripheral blood T cells were cocultured with confluent monolayers of primary, short-term renal cell carcinoma cell lines, a large percentage of the lymphocytes were observed to undergo apoptosis by 72 h. As compared with short-term cultures of NAT, which induced only minimal levels of T cell apoptosis, the two fresh RCCs stimulated an average of 60–70% of the cocultured lymphocytes to stain annexin V/7AAD positive (Fig. 1A). Similar results were obtained with long-term lines. Although a NKE cell line had essentially no apoptogenic effect on cocultured T cells, the long-term RCC cell lines SK-RC-45 and SK-RC-48 induced an average of 40% of the cocultured lymphocytes to undergo apoptosis (Fig. 1, B and C). In addition to annexin V/7AAD staining (42), apoptotic cells can also be discerned by DAPI staining, which reveals the dramatic physical changes, such as nuclear fragmentation and nuclear membrane invagination, that occur within the nuclei of apoptotic cells (31). Evaluation of resting T cells following their coculture with SK-RC-45 revealed a significant increase in the percentage of cells with apoptotic nuclei per visual field compared with cells incubated in medium (Fig. 1D), further supporting the notion that the RCC line SK-RC-45 mediates T cell death by inducing apoptosis.

We previously demonstrated that gangliosides overexpressed by primary RCC tumors (Fig. 2A) and by long-term RCC cell lines (29) participate in T cell apoptosis (29, 30, 38, 43). Our laboratory, as well as others, have also provided evidence that a variety of histologically distinct tumors, including RCC, express FasL. (Fig. 2B), which may also protect tumor cells from immune-mediated elimination by Fas-expressing T cells (12). As a first step in determining whether tumor-associated gangliosides and FasL can independently kill cocultured T cells, and do so by activating distinct apoptotic pathways, SK-RC-45 was pretreated or not with the ganglioside synthase inhibitor PPPP, or anti-FasL Abs before coculture, T cell killing was reduced by 75%, results consistent with the notion that the effect was additive,
respectively. Results represent the mean (±SEM) of three experiments. Lysates were prepared from replicate cells to assess cytochrome c release, as described in Materials and Methods. *p < 0.001 in comparison to medium control; **p < 0.001 in comparison to anti-Fas-treated wild-type Jurkat cells. D. Forty micrograms of the cytoplasmic lysates made from wild-type-, caspase-8-negative-, and FADD-negative Jurkat cells were subjected to Western analysis with Abs to procaspases-8 and -9, to assess the ability of control stimuli of the receptor-dependent and mitochondrial apoptotic pathways to activate those caspases in each cell type. Observed decreases in expression levels of the procaspases following treatment with apoptotic stimuli correspond to their conversion into smaller, active fragments. An anti-actin Ab was used to control for equal loading of protein.

and that two independent pathways were involved. A similar conclusion can be drawn from the experiment presented in Fig. 2D, in which the abilities of rFasL, purified RCC gangliosides, or both were compared with the SK-RC-45 cell line for their individual or collective capacities to kill activated T cells. rFasL and purified RCC gangliosides individually induced 26 and 36% of cocultured activated T cells to undergo apoptosis, respectively, as assessed by TUNEL analysis. When used jointly to treat activated T cells, FasL plus gangliosides induced levels of lymphocyte apoptosis that were approximately the sum of the levels induced by each agent alone, again suggesting that the two act independently and not synergistically. Interestingly, 5-day supernatants from confluent cultures of SK-RC-45 mediated a level of T cell apoptosis that was essentially comparable to that mediated by the combination of FasL plus RCC gangliosides (Fig. 2D). However, the contribution of other tumor-shed compounds cannot be ruled out.

Receptor-dependent apoptosis is initiated by cleavage of procaspase-8, and the intrinsic apoptotic pathway is initiated by activation of caspase-9 (31). When activated T cells were cocultured with confluent monolayers of SK-RC-45, an average of 50% of the lymphocytes became TUNEL positive by 72 h of incubation (Fig. 2E). If the T cells were pretreated 90 min before coculture with inhibitors of caspase-8 or caspase-9, and then again every 24 h during the coincubation, however, tumor-induced apoptosis of the lymphocytes was reduced by 43 and 70%, respectively (Fig. 2E). A similar treatment regimen using a pan caspase inhibitor, however, which inhibits both the death receptor-dependent and -independent pathways, reduced SK-RC-45 stimulated apoptosis by 85%, once more suggesting the likelihood that the extrinsic and intrinsic pathways were mediating their effects independently (Fig. 2E).

Characterization of death receptor-defective caspase-8- and FADD-negative Jurkat cell lines

To more definitively assess the relative contributions of receptor-dependent and -independent pathways to RCC-induced T cell apoptosis, we used two Jurkat cell lines defective in death receptor-dependent apoptosis, both generated using a frameshift mutagen followed by selection with anti-Fas (33, 44). When analyzed for their specific molecular defects by Western analysis, one of these lines was observed to lack procaspase-8, and the other was found to lack the adapter protein FADD (Fig. 3A). Both cell types did express wild-type levels of the FasR, as demonstrated by Western (Fig. 3A) and FACS analysis (Fig. 3B), and additionally synthesized normal levels of procaspases-3 and -9 (data not shown).

Caspase-8- and FADD-negative Jurkat cell clones are resistant to Fas-mediated apoptosis, but retain wild-type susceptibility to apoptosis induced through the mitochondrial pathway

To unravel the molecular mechanism(s) by which RCC tumors mediate T cell apoptosis, it was first necessary to characterize the mutant Jurkat cell clones for their respective sensitivities to prototypic agonists of the receptor- and mitochondrial apoptotic
pathways. Specifically, it was essential to know whether the caspase-8- and FADD-negative Jurkat cell clones retained wild-type levels of susceptibility to the mitochondrial apoptotic pathway, and which caspases were or were not activated when the wild-type and mutant cells were stimulated through either pathway.

To assess whether the mitochondrial apoptotic pathway was actually operative in caspase-8- and FADD-negative Jurkat cells, the wild-type and mutant Jurkat lymphocytic lines were treated with etoposide (10 μg/ml), a well-characterized chemotherapeutic agent known to activate the intrinsic pathway (45). Despite their complete resistance to FasL, both caspase-8- and FADD-negative Jurkat cells displayed essentially wild-type sensitivity to etoposide (Fig. 3C), reflected by the ability of etoposide to induce cytochrome c release from the mitochondria of all three cell types (Fig. 3C). In many Jurkat cell lines, cytochrome c release is also a correlate of effective stimulation of the extrinsic pathway. This is because as "type II" cells, these Jurkat lymphocytes require mitochondrial amplification of death receptor-dependent signals to achieve the threshold of caspase activation requisite for inducing apoptosis (31). Thus, while agonistic anti-Fas Abs induced adequate caspase-8 activation to recruit mitochondria and stimulate cytochrome c release in wild-type Jurkat cells, no such effect was observed in caspase-8- or FADD-negative Jurkat cells (Fig. 3C).

The upstream events occurring or not in the Jurkat cell lines following stimulation with anti-Fas or etoposide are depicted in Fig. 3D. In wild-type Jurkat cells, treatment with either anti-Fas Ab or etoposide led to the activation of both caspases-8 and -9. In contrast, anti-Fas could not activate caspase-9 in mutant cells lacking caspase-8, and could not activate either caspases-8 or -9 in cells lacking FADD. Etoposide did activate caspase-9 in both mutant cell types, and indirectly activated caspase-8 (via activation of caspases-9 and -3; data not shown) in the FADD-negative cells. It was thus clear through both biochemical and functional analyses that the caspase-8- and FADD-negative Jurkat lines would be resistant to strictly receptor-dependent tumor-associated apoptotic

FIGURE 4. FasL and RCC gangliosides independently initiate the MPT through the receptor-dependent and receptor-independent pathways, respectively. Anti-CD3/anti-CD28-activated peripheral blood T cells, wild-type Jurkat cells, caspase-8-negative Jurkat cells, FADD-negative Jurkat cells, and caspase-9 D/N Jurkat cells were cocultured with SK-RC-45 or incubated with RCC-derived gangliosides or rFasL alone or in combination for 18 h. The decrease in mitochondrial potential observed for each lymphocytic cell type following these treatments is reflected in the histograms of DiOC6 fluorescence, which illustrate the mitochondrial potential in control cells (shaded areas) and in the cells exposed to one of the experimental treatments (open areas).
stimuli, while remaining susceptible to products activating the intrinsic pathway.

**FasL and RCC gangliosides independently initiate the mitochondrial permeability transition through the receptor-dependent and -independent pathways, respectively**

The mitochondrial permeability transition which can result either from direct physical or biochemical trauma to mitochondria, or secondarily following death receptor-dependent activation of caspase-8 and cleavage of Bid, involves the formation of nonspecific pores across the inner mitochondrial membrane, leading to cytochrome c release, activation of caspase-9, and apoptosis (31). These changes can be quantified by FACS analysis using positively charged lipophilic, fluorescent probes such as DiOC₆ that are taken up by intact mitochondria, but are less or not accumulated when there is disruption of the mitochondrial inner transmembrane potential related to apoptosis (46). The experiment depicted in Fig. 4 clearly illustrates the impact that the RCC tumor line SK-RC-45 and its tumor-associated, proapoptotic molecules FasL and gangliosides have on inducing an MPT in wild-type Jurkat cells, Jurkat cells compromised in death receptor signaling, and primary T cells. As outlined below, the results suggest that tumor-associated FasL and gangliosides can independently induce the MPT through the receptor-dependent and -independent pathways, respectively, as can the intact SK-RC-45 tumor cells themselves.

Purified RCC gangliosides and rFasL individually induced the MPT in primary, activated T cells, but did so less effectively than if the lymphocytes were treated with both agents together, or if they were coincubated with SK-RC-45 monolayers that synthesized both molecules (Fig. 4). The molecular explanation for these results was revealed by analyzing the responses of wild-type and mutant Jurkat cells to these treatments. As with primary T cells, coincubation with SK-RC-45 or cotreatments with FasL plus RCC gangliosides induced wild-type Jurkat cells to maximal levels of mitochondrial permeability observed. Also like primary T cells, wild-type Jurkat cells underwent a significant MPT in response to gangliosides alone, but the extent of the response was measurably lower than that seen in response to the tumor line or to the combined FasL plus ganglioside treatment. Interestingly, and differentiating the wild-type Jurkat cells from primary, activated T cells, exposure to FasL induced wild-type Jurkat cells to undergo an MPT that was of equivalent magnitude to that induced by gangliosides plus FasL, or by the tumor line, a finding that may relate to the need for mitochondrial participation in receptor-dependent apoptosis of that type II cell: once recruited and amplifying the signal through Fas, additional agonists of the mitochondrial pathway might not be able to enhance the response further.

Further support for the concept that FasL and RCC gangliosides induce apoptosis by independent routes came from examining the MPT responses of the genetically altered Jurkat cell clones to those proapoptotic molecules. rFasL was completely unable to induce an MPT in caspase-8-negative cells, FADD-negative cells, or even in cells rendered caspase-9 deficient by transfection with a dominant-negative construct: in the absence of functional caspase-9, the minimal signal received through the death receptor pathway in these type II cells could not be adequately amplified to stimulate an MPT. Thus, the observation that RCC gangliosides alone could induce the MPT in all three mutant cell types indicated that it was categorically mediating its effects through the intrinsic pathway, in a death receptor-independent fashion.

**FIGURE 5.** SK-RC-45 induces Jurkat cell apoptosis through receptor-dependent and -independent pathways. A, Wild-type, caspase-8 negative, caspase-8 D/N, FADD-negative, and FADD D/N Jurkat cells were coincubated with monolayers of SK-RC-45 as described in Materials and Methods. Following 72 h of coculture, the Jurkat cell populations were isolated and subjected to TUNEL analysis to measure their relative susceptibilities to tumor-induced apoptosis. *, p < 0.001 in comparison to medium; **, p < 0.01 in comparison to wild-type Jurkat cells treated with SK-RC-45 supernatant; ***, p < 0.005 in comparison to wild-type Jurkat cells treated with SK-RC-45 supernatant. B, Wild-type, caspase-8-negative, and FADD-negative Jurkat cells were cocultured with SK-RC-45 for 48h, at which time cell lysates made as described in Materials and Methods were subjected to Western analysis, to assess the tumor-induced activation of proapoptotic molecules in each cell type. Forty micrograms of protein per lane were probed with Abs to Bid, DFF-45, and to procaspases-8 and -9. Observed decrease in the expression levels of Bid and the procaspases following coculture with tumor correspond to their conversion into smaller, active fragments. Disappearance of DFF-45 reflects activation of caspase-3. An anti-actin Ab was used to control for equal loading of protein.

Jurkat cells engineered to be defective in the receptor-dependent apoptotic pathway are partially protected from SK-RC-45-mediated apoptosis: the residual apoptosis observed is mediated by activation of caspase-9 through the intrinsic pathway.

Approximately 51% of wild-type Jurkat cells cocultured for 72 h with the SK-RC-45 cell line were induced to TUNEL positivity (Fig. 5A), which correlated with the complete tumor-induced activation of procaspases-8 and -9, cleavage of Bid, and degradation of DFF-45, the latter a substrate of activated caspase-3. (Fig. 5B). These results were in contrast to those seen for death receptor-defective Jurkat cells, which in the absence of functional caspase-8 or FADD, due either to their deletion (caspase-8- or FADD-negative cells) or incapacitation (caspase-8 D/N or FADD D/N), were ~50% protected from tumor-induced killing (Fig. 5A). The observed protection of the caspase-8- and FADD-negative Jurkat
cells correlated with the significantly reduced capacity of the tumor line to activate procaspase-9, cleave Bid, and degrade DFF-45. That the residual apoptosis observed must have been mediated by a direct effect of a tumor-derived product on the mitochondrion was indicated by the fact that in both mutant cell types with defective death receptor signaling, the tumor line still activated caspase-9 and degraded DFF-45, with only a minimal, indirect effect on procaspase-8 (Fig. 5B). The population of caspase-9 ΔN-transfected Jurkat cells were more completely protected from SK-RC-45-induced apoptosis, reflecting the fact that as type II cells, a caspase-9 deficiency protects against agonists of both apoptotic pathways.

Discussion

Our studies focused on the apoptotic pathways through which the FasL- and ganglioside-overexpressing SK-RC-45 renal cell carcinoma line causes T cell dysfunction, and asked whether both tumor-associated molecules can simultaneously contribute to apoptosis, and if they do so in an additive fashion by activating distinct apoptotic pathways. Although all our functional assays were performed using peripheral blood T cells to demonstrate the physiological relevance of our findings, we also took advantage of genetically altered Jurkat cells to dissect out the apoptotic pathways through which the tumor-associated proapoptotic molecules mediate their effects. Our results implicate caspase-8 and FADD as being important participants in RCC-induced apoptosis of T cells, reflecting in part, their roles in initiating the death receptor-mediated pathway in response to tumor-associated FasL (31, 47, 48). However, the ability of purified RCC gangliosides to stimulate the MPT and TUNEL positivity of primary T cells, and the capacity of the glycosphingolipids to mediate this response even in caspase-8- and FADD-negative Jurkat cells, suggests that RCC tumors can also mediate their apoptotic effects by a receptor-independent mechanism.

The present report demonstrates that a significant component of SK-RC-45-induced T cell apoptosis occurs in a caspase-8- and FADD-dependent fashion, and hence is initiated through a death receptor: short-term and long-term RCC lines express abundant levels of FasL, which in its recombinant form can induce the apoptosis of activated T cells. Additionally, monolayers of SK-RC-45 can induce the apoptosis of primary T cells in vitro by a mechanism that can be 50% inhibited by either pretreating the tumor line with anti-FasL Abs or by treating the T cells with an inhibitor of caspase-8. Furthermore, when coincubated with SK-RC-45, a high percentage of wild-type Jurkat cells are rendered apoptotic, and this is associated with the activation of caspase-8 and cleavage of its downstream substrates (Bid, caspase-9, DFF-45). Conversely, caspase-8-negative Jurkat cells, caspase-8-dominant/negative Jurkat cells, FADD-negative Jurkat cells, and FADD-dominant/negative Jurkat cells are ~50% resistant to SK-RC-45-induced apoptosis, which correlates with the almost complete inability of the tumor line to activate caspase-8 in FADD-negative cells, and the reduced capacity—but not inability—to induce the MPT, caspase-9 activation, Bid cleavage, and DFF-45 breakdown in caspase-8- and FADD-negative cells.

These studies also established that SK-RC-45-induced apoptosis of primary T cells can also occur via a receptor-independent pathway by a mechanism that involves ganglioside-mediated initiation of the MPT. Mitochondrial permeability leads to cytochrome c release, which, once in the cytoplasm, binds dATP and Apaf-1, enabling engagement and activation of procaspase-9 (49, 50). Upon recruitment to the apoptosome, procaspase-3 is activated, which together with other downstream caspases, induce the changes typifying apoptosis (50). The ability of SK-RC-45 to mediate apoptosis in a death receptor-independent manner was revealed by the observation that the tumor cells could induce the MPT and apoptosis in cocultured caspase-8- and FADD-negative Jurkat cells, though the extent of both tumor-induced responses was of significantly less magnitude than those induced in wild-type Jurkat cells or in primary T cells. The likelihood that it was the tumor-associated gangliosides mediating the MPT in the death receptor-defective Jurkat cells was indicated by the fact that purified RCC gangliosides could reproduce the effect. In fact, when the caspase-8-negative Jurkat cells were used as targets, the FasL/ganglioside-expressing SK-RC-45 tumor line had no greater inductive effect on MPT than did the purified RCC gangliosides alone, suggesting that it was the ganglioside component of the tumor cells that was inducing the MPT and apoptosis in those death receptor-crippled cells. That the SK-RC-45-mediated MPT observed in caspase-8- and FADD-negative Jurkat cells did progress to direct activation of the intrinsic apoptotic pathway and subsequent apoptosis was indicated by the patterns of caspase activation and substrate degradation observed in those cell types following coculture with tumor. So while there were maximal levels of apoptosis and complete cleavage of procaspases-8, -9, Bid, and DFF-45 in the wild-type Jurkat cells cocultured with tumor, the receptor-independent mechanism by which SK-RC-45 killed fewer caspase-8- and FADD-negative Jurkat cells correlated with significant but lesser levels of procaspase-9 activation, Bid cleavage and DFF-45 degradation observed in those lymphocytes in the absence of caspase-8 activation.

The Jurkat cells used in these studies require the mitochondrial pathway to amplify apoptotic signals received through the TNF-like receptors. In such “type 2” cells, the levels of active caspase-8 accumulated following receptor ligation are inadequate to activate caspase-3, but are sufficient to cleave the proapoptotic Bcl-2 family member Bid into a truncated form that can mediate cytochrome c release and subsequent caspase-9 activation (48, 51). The results presented here indicate that the mitochondrial amplification pathway is involved in death receptor-dependent, RCC-induced killing of Jurkat cells, as has been shown previously for squamous cell carcinoma of the head and neck (46). Consistent with this hypothesis was our finding that while rFasL could induce an MPT in wild-type Jurkat cells, and as expected, could not initiate that process in caspase-8- or FADD-negative Jurkat cells, it also could not induce an MPT in caspase-9 ΔN-transfected Jurkat cells—despite the fact that in the latter cells, the upstream components of the death receptor pathway are intact. These results support the concept that so little caspase-8 is activated through the death receptor pathway in these type II Jurkat cells, that several rounds of mitochondrial amplification requiring caspase-9 activation are required to mediate a measurable response. It is also interesting to note that rFasL alone stimulated a level of MPT in wild-type Jurkat cells that was equivalent to that induced by rFasL plus gangliosides. In these cells it is likely that, once recruited to amplify receptor-dependent apoptosis, mitochondria are fully integrated into the process, and unable to participate any further by additional, direct, receptor-independent insults. In fact, the finding that treatment with rFasL plus gangliosides did have an additive effect on MPT in primary T cells supports the notion that, unlike the type II Jurkat cells used in these studies, primary, activated T lymphocytes are type I cells (52), which can be killed by RCC tumor cells through both apoptotic pathways simultaneously. Indeed, because RCC tumor cells are heterogeneous with respect to their elaboration of TNF, gangliosides (29), and FasL (7), it is likely that they can initiate the apoptosis of contiguous T lymphocytes through either the extrinsic or intrinsic pathways, or both, depending on the levels
and which proapoptotic molecule(s) the tumor cells are synthesiz-
ing. As type I cells, the lymphocytes can be killed through the Fas pathway directly, without mitochondrial amplification, and hence regardless of whether the T cells are expressing elevated levels of the antia apoptotic Bcl-2 or Bcl-xL proteins (31). Similarly, because gangliosides can inhibit NF-κB activation (28), a reduced expression of NF-κB-dependent antia apoptotic proteins can render lymphocytes susceptible to ganglioside-mediated reactive oxygen species production, cytochrome c release, and caspase-9 activation, and hence apoptosis through the intrinsic apoptotic pathway (30).

Our findings show that RCC tumors can induce apoptosis of primary T cells through two independent apoptotic pathways, by synthesizing products that can activate, simultaneously and in an additive fashion, the receptor-dependent and -independent pathways. Although previous studies demonstrated that the apoptosis of T cells mediated by squamous cell carcinoma of the head and neck is initiated by receptor-dependent signals but significantly amplified by a mitochondrial loop (46), we extend these studies by demonstrating the ability of RCC-associated gangliosides to stimulate apoptosis in a strictly death receptor-independent fashion by directly inducing the MPT. These results would suggest that mitochondria are not only involved in amplifying tumor-derived apoptotic signals initiated through the receptor-mediated pathway, but can also be directly recruited by tumor products to release cytochrome c, activate caspase-9, and hence initiate apoptosis through the intrinsic pathway. This mitochondrial pathway of tumor-induced T cell apoptosis thus qualifies as an additional therapeutic target for enhancing immune-based antitumor therapies.

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