The Bcl-2 Transgene Protects T Cells from Renal Cell Carcinoma-mediated Apoptosis

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ABSTRACT

Purpose: Tumors induce T-cell apoptosis as a mechanism of inhibiting antitumor immunity. Using coculture experiments, it has been shown that tumor lines stimulate T-cell apoptosis by a pathway involving a mitochondrial permeability transition and cytochrome c release. Activated T cells express abundant levels of Bcl-2, an antiapoptotic molecule that would be expected to confer resistance to such tumor-mediated killing. We examined the mechanism by which Bcl-2 is dysregulated in T cells exposed to the renal tumor line SK-RC-45, and we determined whether overexpressing Bcl-2 protects T cells from tumor-mediated apoptosis.

Experimental Design: Activated T lymphocytes and Jurkat cells transfected or not transfected with Bcl-2 were exposed to SK-RC-45 for 48–72 h. After coculture, lymphocytes were analyzed for Bcl-2 expression using Western analysis and for tumor-induced apoptosis by terminal deoxynucleotidyl transferase-mediated nick end labeling. The role of SK-RC-45-stimulated caspase activation in degrading T-cell Bcl-2 was assessed using a pan-caspase inhibitor, as well as a specific inhibitor of caspase-9.

Results: The renal cell carcinoma cell line SK-RC-45 sensitizes peripheral blood activated T lymphocytes and Jurkat cells to apoptosis by a mechanism that involves degradation of the antiapoptotic protein Bcl-2. The SK-RC-45-induced modulation of lymphocyte Bcl-2 levels was largely caspase independent because pretreatment of T cells with pan-caspase inhibitor III or an inhibitor of caspase-9 had minimal or no effect on stabilizing the protein, although it did provide protection against apoptosis. Overexpression of Bcl-2 protected Jurkat cells from tumor-mediated killing.

Conclusions: Bcl-2 inhibition is a mechanism by which tumors may render lymphocytes sensitive to other tumor-derived, proapoptotic stimuli.

INTRODUCTION

There is ample evidence to suggest that the adaptive immune system can generate protective responses to developing tumors (1, 2). Indeed, tumor-specific antigens have been identified for a variety of malignancies (3–5), and cancer patients often possess enhanced numbers of circulating CD8+ T lymphocytes with specificity for those tumor-derived peptides (6). However, although significant numbers of inflammatory cells within malignant organs indicate that immune cells are homing to involved tissues to initiate antitumor responses (7, 8), the continued, progressive growth of tumors in these patients attests to the generally ineffective nature of these reactions (9, 10). Among the mechanisms endowing tumors with the capacity to evade the immune system are inhibited class I expression (11), aberrant antigen processing and/or presentation (12, 13), and to enhance cytokine production favoring immunosuppressive T(II) responses (10). Recent studies from a number of laboratories, including our own, now suggest that a variety of tumors also elaborate factors that induce the apoptosis of responding inflammatory lymphocytes within malignant organs indicate that immune cells are homing to involved tissues to initiate antitumor responses (7, 8), the continued, progressive growth of tumors in these patients attests to the generally ineffective nature of these reactions (9, 10). Among the mechanisms endowing tumors with the capacity to evade the immune system are inhibited class I expression (11), aberrant antigen processing and/or presentation (12, 13), and mutations in the caspase cascade that confer resistance to cytotoxic molecules secreted by host effector cells (14). Tumors have also been shown to inhibit the expression of T-cell receptor signaling components (15) and to enhance cytokine production favoring immunosuppressive T(II) responses (10). Recent studies from a number of laboratories, including our own, now suggest that a variety of tumors also elaborate factors that induce the apoptosis of responding inflammatory lymphocytes (16–19). Indeed, in situ TUNEL analysis has revealed that 10–15% of T cells infiltrating histologically distinct tumors, including RCC, are apoptotic (16, 20). These effects extend into the periphery, as a subset of circulating lymphocytes from cancer patients is often either apoptotic (21) or sensitized to activation-induced cell death (16).

Studies are in progress to identify the tumor-derived molecules mediating these apoptogenic effects and the molecular...
pathways through which those death signals are being transduced in T cells (22, 23). A subset of ligands belonging to the TNF family has been implicated in tumor-mediated T-cell death (16–19), as has the need for a mitochondrial cascade to amplify those receptor-dependent signals (23). A more recent report from our laboratory indicated that tumor-derived gangliosides also play a significant role in mediating the apoptogenic effects of a RCC tumor on activated, peripheral blood T cells because death of the cocultured lymphocytes was reduced by approximately 50% if the SK-RC-45 tumor cell line was first pretreated overnight with the glucosylceramide synthase inhibitor PPPP (24). The contribution of gangliosides to T-cell apoptosis may result from a direct effect of the tumor-derived glycosphingolipids on mitochondrial membrane permeability and cytochrome c release, as has been demonstrated for GD3 and GM1 on hepatocyte mitochondria (25). Accompanying SK-RC-45-induced T-cell apoptosis was a tumor-mediated reduction in lymphyme Bcl-2 expression levels, which was also abrogated by blocking ganglioside expression on the tumor line (24). Bcl-2 is involved in protecting mitochondria from reactive oxygen species accumulation (26), disruption of transmembrane potential (27), and cytochrome c release (28). Here we show that the tumor-induced loss of Bcl-2 is mediated by two mechanisms: (a) a major caspase-independent pathway; and (b) a minor caspase-dependent pathway. The importance of Bcl-2 in regulating the sensitivity of T cells to RCC-induced apoptosis is demonstrated by the fact that overexpression of Bcl-2 protects the lymphocytes from SK-RC-45-mediated apoptosis by a mechanism that involves abrogating cytochrome c release and activation of caspases-9 and -3.

MATERIALS AND METHODS

Reagents. Agonistic anti-Fas Ab (IgM, CH-11 clone) was purchased from Upstate Biotechnology (Lake Placid, NY) and used at a concentration of 10 ng/ml to induce apoptosis in Jurkat cells. A polyclonal rabbit anti-caspase-3 Ab recognizing the active 12- and 17-kDa fragments was purchased from BD PharMingen (San Diego, CA) and used at 2 μg/ml. A murine monoclonal Ab to caspase-9, recognizing both the pro-form (46 kDa) and its active fragments (35/37 kDa), was from Oncogene (Cambridge, MA) and used at 10 ng/ml to induce apoptosis in lymphocytes. The Ab to cytochrome c, a major caspase-independent pathway; and (b) a minor caspase-dependent pathway. The importance of Bcl-2 in regulating the sensitivity of T cells to RCC-induced apoptosis is demonstrated by the fact that overexpression of Bcl-2 protects the lymphocytes from SK-RC-45-mediated apoptosis by a mechanism that involves abrogating cytochrome c release and activation of caspases-9 and -3.

Plasmids and Transfection. A FLAG-tagged cDNA encoding human Bcl-2 was a generous gift from Dr. Gabriel Nunez, University of Michigan Medical School (29). The insert was subcloned into PCDNA3, and aliquots of 10 million wild-type Jurkat cells were transfected with 10 μg of the plasmid DNA using a BTX ElectroSquarePorator T820 electroporator set to give a pulse of 230 V for 65 ms or a field strength of 0.575 kV/cm, as recommended by the Electronic Genetics Division of BTX (San Diego, CA).

Peripheral Blood T Cells and Cell Lines. Peripheral blood was obtained from healthy volunteers with informed consent. Blood was centrifuged over a Ficoll-Hypaque density gradient (Amersham Pharmacia Biotech AB, Uppsala, Sweden) to obtain total leukocytes, from which T cells were purified by negative magnetic selection using microbeads coated with Abs to CD14 (macrophages), CD16 (natural killer cells), CD19 (B cells), CD56 (natural killer cells), and glycophorin A (RBCs; Stem Cell Technologies, Vancouver, Canada). The T-cell isolation procedure yielded cells that were >95% positive for CD3 as defined by immunocytometry.

Stimulation of T cells with cross-linked anti-CD3 Ab (OKT3) plus anti-CD28 Ab was performed by first coating flasks with 10 μg/ml anti-CD3 and 5 μg/ml anti-CD28 in 1× Tris buffer (pH 8.0) for 1 h. The flasks were washed twice with RPMI 1640 to remove unbound Abs, and cells were added to a density of 1 × 10^6 cells/ml for stimulation. Cells were activated for 3 days, at which point lymphocytes were transferred to fresh flasks, in which they were expanded for 2–3 weeks in the presence of 200 units/ml IL-2 and restimulated once with anti-CD3/anti-CD28 before use.

The Jurkat leukemic T-cell line was purchased from American Type Culture Collection (Manassas, VA) and maintained in complete medium (RPMI 1640; BioWhittaker, Walkersville, MD) supplemented with 10% FCS (HyClone, 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, Inc., Grand Island, NY). Wild-type Jurkat cells were transfected with a plasmid encoding the anti-apoptotic molecule, Bcl-2. Bulk transfected populations were selected using G418, and cells surviving this treatment were cloned and assessed for Bcl-2 expression by Western analysis. The well-characterized long-term RCC line, SK-RC-45 (30), was obtained from Dr. Neil Bander (The New York Hospital, Cornell University Medical College). The RCC line was maintained in complete medium at 37°C with 5% CO2 and allowed to reach confluence in 150-mm dishes before use in coculture experiments with Jurkat cell populations. A normal human colon smooth muscle cell line (SMC) was obtained from the American Type Culture Collection, grown in complete media, and used as a negative control in coculture experiments.

Induction of Apoptosis in T Lymphocytes and in Jurkat Cell Lines. The ability of tumor cells to induce apoptosis was assessed by incubating 150-mm tissue culture dishes containing confluent tumor cells with 8 × 10^6 normal peripheral blood T cells, wild-type Jurkat cells, or Bcl-2-overexpressing Jurkat cells at a tumor cell:T-cell ratio of 1:1. After coculture, lymphocytes were harvested by gentle washing to detach them from adherent SK-RC-45 and SMC monolayers, at which time they were processed for either TUNEL analysis or isolation of whole cell lysates.

Analysis of DNA Fragmentation by TUNEL Assay. Cells were fixed in 1% paraformaldehyde and stained and analyzed for apoptosis using the APO-BRDU system (Phoenix Flow Systems, San Diego, CA). Briefly, cells were labeled with 50 μl of DNA solution containing 10 μl of terminal deoxynucleotidyl transferase reaction buffer. Cells were rinsed and resus-
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Solution with 0.1% Tween (TBST) and subsequently probed incubation with 5% nonfat dry milk in Tris Boric Acid Saline described previously (31), the blots were blocked by overnight membranes by electroblotting (Bio-Rad, Richmond, CA) as °C until use. Equivalent amounts of protein were mixed cytoplasmic extracts were collected, aliquoted, and stored at vortexed vigorously and centrifuged at maximum speed, and the base (pH 7.4); Sigma] to the pellets, Eppendorf tubes were once with ice-cold mitochondria isolation buffer [20 m M phenylmethylsulfonyl fluoride, 100 μg/ml pefabloc, and 100 μg/ml chymostatin] in a final volume of 150 μl for 15 min at 4°C. After adding 10 μl of a 10% NP40 solution [20 m M Tris base (pH 7.4); Sigma] to the pellets, Eppendorf tubes were vortexed vigorously and centrifuged at maximum speed, and the cytoplasmic extracts were collected, aliquoted, and stored at −80°C until use. Equivalent amounts of protein were mixed with an equal volume of 2× Laemmli buffer, boiled, and resolved on 12% SDS-PAGE gels. After transfer to nitrocellulose membranes by electroblotting (Bio-Rad, Richmond, CA) as described previously (31), the blots were blocked by overnight incubation with 5% nonfat dry milk in Tris Boric Acid Saline Solution with 0.1% Tween (TBST) and subsequently probed with the specific primary Abs described above. The immunoreactive proteins were visualized using horseradish peroxidase-linked secondary Abs and enhanced chemiluminescence (ECL Western blotting kit; Amersham).

Cytochrome c Assay. For analysis of cytochrome c release from target cell mitochondria after coculture of the lymphocytes with tumor cell monolayers, 1 × 10^7 lymphocytes were spun down, washed once with ice-cold PBS, and washed once with ice-cold mitochondria isolation buffer [20 m M HEPES-KOH, 10 m M KCl, 1.5 m M MgCl2, 1 m M EGTA, and 250 m M sucrose, containing protease inhibitors (5 μg/ml apro- tin, 2 μg/ml leupeptin, 1 m M phenylmethylsulfonyl fluoride, 100 μg/ml pefabloc, and 100 μg/ml chymostatin)]. The cell pellet was resuspended in 100 μl of mitochondrial isolation buffer and, after 20 min on ice, homogenized with a Dounce homogenizer. The homogenate was centrifuged at 750 × g for 20 min, and the supernatant, which contained released cytochrome c, was assayed for protein concentration. Equivalent amounts of protein were then mixed with an equal volume of 2× Laemmli buffer, boiled, resolved on 12% SDS-PAGE gels, and processed for Western blot analysis using a murine polyclonal anti-cytochrome c Ab as described above.

RESULTS

Tumor-induced Apoptosis of Activated T Cells Coincides with Reduced Expression of Bcl-2. Previous studies from this and other laboratories have demonstrated that tumor cell lines can induce T-cell apoptosis in coculture experiments (16–18, 24). Although tumors have been shown capable of initiating this process through both receptor-dependent and receptor-independent signaling pathways, the type II nature of Jurkat cells and T lymphocytes (32, 33) makes activation of the mitochondrial permeability transition requisite to apoptosis induced by either mechanism. This is because cytochrome c release provides the needed amplification for receptor-mediated signals, and it also initiates the caspase cascade when apoptotic stimuli are received by the mitochondrion directly (25, 34). Here we show that at a time when anti-CD3/anti-CD28 expanded T cells are resistant to apoptosis mediated by soluble anti-Fas Ab (Fig. 1A), cross-linked anti-Fas Ab (Fig. 1A), or exposure to soluble FasL (data not shown), they are sensitive to tumor-mediated apoptosis induced by a 72-h coculture with SK-RC-45. The ability of activated T cells to resist anti-Fas-mediated killing may be attributable to the elevated levels of Bcl-2 in the activated T cells (35). The sensitivity of the activated T cells to tumor-induced apoptosis, on the other hand, is likely explained by the Western blot depicted in Fig. 1B, which shows that the comparatively abundant expression levels of Bcl-2 characterizing activated T cells maintained in media were.

![Figure 1](https://example.com/f1.png)

**Fig. 1.** The RCC line SK-RC-45 inhibits Bcl-2 expression by activated, peripheral blood T cells and induces lymphocyte apoptosis. A, peripheral blood T cells were activated with anti-CD3/anti-CD28 for 3 days before expansion in 200 units/ml IL-2 and a second stimulation just before use. Replicate aliquots of these activated lymphocytes were not coincubated or coincubated for 72 h with anti-Fas, cross-linked anti-Fas, or SK-RC-45 at a tumor:T-cell ratio of 1:1 before assessing the T-cell populations for apoptosis by TUNEL. The results presented for SK-RC-45 and soluble anti-Fas are the mean ± SD of four separate experiments. The results for cross-linked anti-Fas are representative of two individual experiments. B, cell lysates made from peripheral blood T cells, activated as described in A and coincubated or not coincubated with SK-RC-45 for 72 h at a tumor cell:T-cell ratio of 1:1, were assessed for Bcl-2 expression levels by Western analysis, using 20 μg protein/ lane. Results are representative of four separate experiments.
reduced significantly when the lymphocytes were coincubated for 72 h with the tumor cell line. This tumor-mediated reduction in Bcl-2 accumulation was accompanied by an induction of apoptosis, as measured by TUNEL analysis, which in four experiments affected an average of 36% of the T cells by 72 h of coculture (Fig. 1A). Similar results were obtained when the extent of tumor-induced T cell killing was assessed by trypan blue exclusion (data not shown).

A similar relationship between tumor-induced apoptosis and reduced Bcl-2 expression levels held for Jurkat T cells. When Jurkat cells were coincubated with SK-RC-45 for 48 h at a ratio of 1:1, an average of 59% of the cocultured lymphocytes were found to contain DNA breaks when assessed by TUNEL analysis (Fig. 2A; n = 3). This is in comparison with an average of 4% of Jurkat cells that became apoptotic after coincubation with smooth muscle cells or in media alone (Fig. 2A). As in the case of the normal, activated peripheral blood T cells, tumor-induced apoptosis of Jurkat cells was accompanied by a decrease in Bcl-2 expression by the lymphocytes, which was dramatically inhibited by 48 h of coincubation (Fig. 2B). The expression of Bcl-2 by Jurkat cells remained elevated in media controls (Fig. 2B) and when those lymphocytes were cocultured with smooth muscle cells (data not shown).

Previous studies indicate that Bcl-2 is a substrate for caspase-3. A caspase-mediated decrease in Bcl-2 expression would imply that Bcl-2 degradation occurs subsequent to activation of the receptor or mitochondrial apoptotic pathway and that Bcl-2 degradation may serve to amplify an already initiated apoptotic process. On the other hand, a caspase-independent mechanism for Bcl-2 degradation might suggest that Bcl-2 decay is induced by tumor in an earlier time frame than it activates the caspase cascade, and hence loss of the antiapoptotic protein might sensitize cells to subsequent apoptotic stimuli. To assess whether altered Bcl-2 expression by tumor-exposed lymphocytes occurs by a caspase-dependent or -independent mechanism, Jurkat cells were coincubated with SK-RC-45 in the presence of either pan-caspase inhibitor III (150 μM) or caspase-9 inhibitor II (150 μM). After coculture, cell lysates made from Jurkat cells isolated from the monolayers were subjected to Western analysis using an Ab to Bcl-2 to assess the ability of caspase inhibitors to abrogate tumor-induced Bcl-2 disappearance. Abs to actin were used to control for equal protein loading. Results are representative of multiple separate experiments. B, Jurkat cells coincubated with SK-RC-45 for 48 h in the presence or absence of caspase inhibitors described above were isolated from the RCC monolayers and assessed for tumor-induced apoptosis by TUNEL. Results are representative of multiple separate experiments.
tured with the RCC tumor line. A specific inhibitor of caspase-9 was unable to reverse this decline, and pan-caspase inhibitor III had only a minimal effect. The results suggest that tumor-induced modulation of Bcl-2 expression is largely caspase independent. Interestingly, whereas only a minor component of Bcl-2 down-regulation was caspase dependent, pan-caspase inhibitor III almost completely blocked apoptosis in the same experiment. Indeed, the specific inhibitor of caspase-9 reduced SK-RC-45-mediated apoptosis by approximately 50% (Fig. 3A), although it had virtually no ability to reverse tumor-mediated Bcl-2 degradation (Fig. 3A). Thus, a major component of tumor-induced Bcl-2 loss from lymphocytes is seemingly upstream of and not dependent on tumor-induced caspase activation and apoptosis.

**Overexpression of Bcl-2 Protects Jurkat T Cells from Tumor-induced Apoptosis.** As a protein whose antiapoptotic effects are exerted by protecting mitochondria from both oxidative stresses and the multiple proapoptotic molecules that can lead to cytochrome c release and caspase activation (26, 36), inhibition of Bcl-2 expression by tumors would be expected to sensitize or induce T cells to apoptosis. Because Jurkat cells, like activated, normal peripheral blood T cells, are susceptible to SK-RC-45-induced apoptosis (16), the ease with which T-cell line can be transfected afforded the opportunity to inquire into the potential, protective effect of Bcl-2 overexpression in this setting. A 5′FLAG-tagged Bcl-2 cDNA was cloned into the EcoRI site of pcDNA3, which was then used to transfect Jurkat T cells. G418-resistant, Bcl-2-transfected Jurkat cells were cloned by limiting dilution, and several such isolates were shown to be resistant to etoposide (10 μg/ml) and anti-Fas Ab (10 ng/ml), in contrast to wild-type Jurkat cells and those expressing the empty pcDNA3 vector, which were sensitive to both apoptotic stimuli (Fig. 4A). Western analysis performed on a FLAG-Bcl-2-transfected clone revealed two protein bands recognized by the anti-Bcl-2 Ab: a native molecule also present in lysates from wild-type cells; and an additional, appropriately higher molecular mass protein specific to the transduced cells (Fig. 4B). Indeed, when lysates from control and Bcl-2-transfected cells were subjected to Western analysis using an Ab to the FLAG peptide, only a single band from the Bcl-2-transduced clone alone was recognized, representing the FLAG-tagged transgene (Fig. 4B). Thus, it was clear that the G418-resistant, Bcl-2-transfected clone overexpressed the antiapoptotic protein and was protected from both receptor and mitochondrial pathways of apoptosis.

To assess the possibility that Bcl-2 overexpression would confer Jurkat cells protection from SK-RC-45-mediated apoptosis, wild-type and Bcl-2-transfected Jurkat cells were analyzed for DNA breaks by TUNEL after a 48-h cocultivation with monolayers of SK-RC-45. Multiple experiments demonstrated that wild-type Jurkat cells were highly sensitive to the tumor line, with an average of 42% of the cocultured lymphocytes testing positive for apoptosis (Fig. 5). Unlike wild-type cells, however, Bcl-2-transfected cells were only minimally affected, with an average of only 3% of the transfused cells succumbing to a tumor-induced apoptotic death (Fig. 5). Similar results were obtained upon assessing T-cell viability by trypan blue dye exclusion (data not shown).

The fact that Bcl-2 overexpression provided Jurkat cells with significant protection against SK-RC-45-mediated-apoptosis led us to ask what specific proapoptotic events induced by the tumor line in wild-type cells were abrogated by constitutive expression of the antiapoptotic molecule in the transfected lymphocytes. To this end, lysates made from tumor-exposed wild-type and Bcl-2-overexpressing cells were subjected to Western analysis using Abs to cytochrome c and the active fragments of caspases-9 and -3. As demonstrated in Fig. 6, tumor cells induced the release of cytochrome c from the mitochondria of coincubated wild-type Jurkat cells, an event associated with the coincident activation of caspases-9 and -3 in those lymphocytes. Indeed immunoblotting revealed the presence of active fragments for caspase-9 (35/37 kDa) and caspase-3 (12/17 kDa) in wild-type Jurkat cells cocultured with SK-RC-45. Unlike wild-type cells, on the other hand, Jurkat cells overexpressing Bcl-2 were completely resistant to SK-RC-45-mediated cytochrome c release and caspase-9 and -3 activation, demonstrating both the importance of a tumor-induced, mitochondrial permeability transition to RCC-mediated apoptosis of T cells and the mechanism by which the Bcl-2 transgene inhibits the killing (Fig. 6).
Fig. 5 A Jurkat cell clone overexpressing Bcl-2 is resistant to SK-RC-45-mediated apoptosis. Wild-type and Bcl-2-overexpressing Jurkat cells were each cocultured or not cocultured with SK-RC-45 cells at a 1:1 ratio for 48 h before being isolated from the monolayers and assessed for tumor-induced apoptosis by TUNEL analysis. Results are the mean ± SD of four separate experiments.

Fig. 6 Overexpression of the Bcl-2 transgene protects Jurkat cells from tumor-induced activation of the caspase cascade. Wild-type and Bcl-2-overexpressing Jurkat cells were cocultured or not cocultured with SK-RC-45 for 48 h. T cells were collected after the coincubation and used to make lysates, as described in “Materials and Methods,” that were subjected to Western analysis to assess tumor-induced cytochrome c release and caspase-9 and -3 activation. The active caspase-9 fragments run at 35/37 kDa, and the active caspase-3 fragments run at 12/17 kDa. Cytochrome c is a 15-kDa protein. The results are representative of 10 separate experiments.

DISCUSSION

The results presented here touch on the mechanism by which some tumors are able to induce apoptosis of activated T lymphocytes, cells that characteristically express augmented levels of the antiapoptotic protein Bcl-2 (35). Previous studies from multiple laboratories have convincingly demonstrated the ability of various tumors to mediate T-cell apoptosis. Most of the tumors described in those reports expressed elevated levels of the TNF-related ligands TNF-related apoptosis-inducing ligand (TRAIL), FasL, TNF, or CD70, and hence purportedly killed T cells in a receptor-dependent fashion (18–20, 37). However, Gastman et al. (23) extended these studies by showing that pretreatment of Jurkat cells with inhibitors of the mitochondrial permeability transition or caspase-9 activity protected them from apoptosis induced by a FasL-expressing SCHHN. This result suggested the importance of the mitochondrial amplification loop in transducing the initial SCHHN-mediated apoptotic signal received by Jurkat cells through Fas. We recently showed that in addition to killing T cells by a receptor-dependent mechanism, gangliosides synthesized by SK-RC-45 played an integral role in the ability of that tumor line to mediate T-cell apoptosis (24). This conclusion was based on the capability of the ganglioside synthesis inhibitor PPPP to reverse approximately 50% of the apoptotic effect and the capacity of gangliosides isolated from either the SK-RC-45 cell line or RCC tumor explants to mediate comparable T-cell killing. The involvement of mitochondria in ganglioside-mediated T-cell apoptosis was demonstrated by showing that pretreatment of SK-RC-45 with PPPP inhibited not only the ganglioside synthesis and apoptogenicity of the tumor cells, but also their ability to induce cytochrome c release from T-cell mitochondria (24). That such RCC ganglioside-mediated apoptosis may occur via a direct effect on mitochondria is suggested by previous reports examining the effects of ceramide and GD3 on hepatocytes (25).

In our studies, we noted that T cells activated by anti-CD3/anti-CD28 Abs and expanded in IL-2 with periodic restimulation developed into a population resistant to Fas-mediated apoptosis. These cells, a mixture of CD4+ and CD8+ T lymphocytes, remained resistant to killing through Fas for 2–3 weeks, even though they expressed high levels of the receptor (16). Others have similarly reported the resistance of anti-CD3-activated peripheral blood T cells and antigen-primed cells to Fas-mediated apoptosis (38, 39). The ability of SK-RC-45 to kill these same cells suggests that the tumor line can initiate apoptosis of activated T cells by a Fas-independent pathway, an idea consistent with our data demonstrating that tumor-derived gangliosides are involved in the process.

Here we show that SK-RC-45-induced apoptosis of both activated, peripheral blood T cells and Jurkat cells is accompanied by a reduction of Bcl-2 expression by the lymphocytes and that overexpression of Bcl-2 protects the T cells from tumor-induced death. The fact that the ratio of CD4+ : CD8+ T lymphocytes remains relatively constant before and after coculture with the tumor suggests that the two cell types are equally susceptible to SK-RC-45-mediated apoptosis (data not shown), a finding consistent with the report that CD4+ and CD8+ T cells express equivalent levels of Bcl-2 (40, 41). The primary function of Bcl-2 is to maintain the integrity of the mitochondrion, an organelle whose pivotal importance in the two major pathways of programmed cell death arises from its ability to release a variety of apoptogenic molecules sequestered within its membranes (42). A diverse array of proapoptotic members of the Bcl-2 protein family can be activated by intracellular signaling events or environmental stresses to initiate this chain of events. One example is the activation of Bid to tBid after TNF-mediated caspase-8 activation (43–45). Another is the
activation of BAD, which occurs upon withdrawal of growth factors (46). tBid proteolytically activates two other proapoptotic Bcl-2 family members, Bax and Bak; these oligomerize and form pores that permeabilize mitochondrial membranes, allowing release of the caspase-9 activator, cytochrome c (47). In type II cells, where adequate levels of caspase-8 are not generated at the death-inducing signaling complex (DISC) to cleave procaspase-3 and induce the death pathway directly, this scheme for amplifying receptor-mediated signals is requisite for apoptosis to ensue after receptor stimulation (48). Bcl-2, when available in excess, protects the cells from this apoptotic death by heterodimerizing with tBid and inhibiting its activation of Bax and Bak (49), whereas molecules such as BAD and Bik mediate a proapoptotic effect, in turn, by inactivating Bcl-2 (47). Thus, it is the relative expression levels of the pro- and anti-apoptotic proteins, characterizing a cell in specific stages of activation or differentiation, that govern its susceptibility or resistance to apoptotic stimuli (36). So whereas the enhanced Bcl-2 expression typifying activated T cells may explain the resistance of those lymphocytes to the death ligands and activated caspases simultaneously induced in T cells after stimulation, it is the ability of SK-RC-45 to inhibit lymphocyte Bcl-2 expression that likely sensitizes activated T cells and Jurkat cells to tumor-derived apoptogenic molecules. This hypothesis is supported by our observations that Bcl-2 expression declines in T cells cocultured with ganglioside-synthesizing SK-RC-45 cells in a time frame that precedes apoptosis and that Jurkat cells overexpressing the Bcl-2 transgene are protected from a tumor-mediated, caspase-dependent death (24).

In the studies reported by Gastman et al. (23), inhibitors of the mitochondrial pathway prevented etoposide-mediated apoptosis but had no significant inhibitory effect on apoptosis initiated through the Fas receptor. This is consistent with results expected for the type I Jurkat cells, used by the authors, which do not require mitochondrial amplification of apoptotic signals received through TNF-related death receptors (48). The authors thus found it enigmatic that mitochondrial permeability transition (MPT) inhibitors could abrogate ostensibly Fasl-dependent SCHHN-mediated killing of these type I Jurkat cells because cyclosporin A or bongkrekic acid could not prevent anti-Fas Ab-mediated killing of the identical target cells (23). They reconcile this discrepancy by suggesting that Fas signaling in the tumor microenvironment is potentially weaker than that delivered by direct cross-linking of Fas by anti-Fas Abs and hence requires mitochondrial amplification. Although the ability of cyclosporin A and bongkrekic acid to inhibit SCHHN-mediated killing of Jurkat cells because cyclosporin A or bongkrekic acid could not prevent anti-Fas Ab-mediated killing of the identical target cells (23). They reconcile this discrepancy by suggesting that Fas signaling in the tumor microenvironment is potentially weaker than that delivered by direct cross-linking of Fas by anti-Fas Abs and hence requires mitochondrial amplification. Although the ability of cyclosporin A and bongkrekic acid to inhibit SCHHN-mediated killing of Jurkat cells because cyclosporin A or bongkrekic acid could not prevent anti-Fas Ab-mediated killing of the identical target cells (23). They reconcile this discrepancy by suggesting that Fas signaling in the tumor microenvironment is potentially weaker than that delivered by direct cross-linking of Fas by anti-Fas Abs and hence requires mitochondrial amplification. Although the ability of cyclosporin A and bongkrekic acid to inhibit SCHHN-mediated killing of Jurkat cells because cyclosporin A or bongkrekic acid could not prevent anti-Fas Ab-mediated killing of the identical target cells (23). They reconcile this discrepancy by suggesting that Fas signaling in the tumor microenvironment is potentially weaker than that delivered by direct cross-linking of Fas by anti-Fas Abs and hence requires mitochondrial amplification. Although the ability of cyclosporin A and bongkrekic acid to inhibit SCHHN-mediated killing of Jurkat cells because cyclosporin A or bongkrekic acid could not prevent anti-Fas Ab-mediated killing of the identical target cells (23). They reconcile this discrepancy by suggesting that Fas signaling in the tumor microenvironment is potentially weaker than that delivered by direct cross-linking of Fas by anti-Fas Abs and hence requires mitochondrial amplification. Although the ability of cyclosporin A and bongkrekic acid to inhibit SCHHN-mediated killing of Jurkat cells because cyclosporin A or bongkrekic acid could not prevent anti-Fas Ab-mediated killing of the identical target cells (23). They reconcile this discrepancy by suggesting that Fas signaling in the tumor microenvironment is potentially weaker than that delivered by direct cross-linking of Fas by anti-Fas Abs and hence requires mitochondrial amplification. Although the ability of cyclosporin A and bongkrekic acid to inhibit SCHHN-mediated killing of Jurkat cells because cyclosporin A or bongkrekic acid could not prevent anti-Fas Ab-mediated killing of the identical target cells (23). They reconcile this discrepancy by suggesting that Fas signaling in the tumor microenvironment is potentially weaker than that delivered by direct cross-linking of Fas by anti-Fas Abs and hence requires mitochondrial amplification. Although the ability of cyclosporin A and bongkrekic acid to inhibit SCHHN-mediated killing of Jurkat cells because cyclosporin A or bongkrekic acid could not prevent anti-Fas Ab-mediated killing of the identical target cells (23). They reconcile this discrepancy by suggesting that Fas signaling in the tumor microenvironment is potentially weaker than that delivered by direct cross-linking of Fas by anti-Fas Abs and hence requires mitochondrial amplification.

We also find that SK-RC-45 monolayers inhibit Bcl-2 expression levels within cocultured Jurkat cells and activated T lymphocytes, in parallel with the ability of the tumor line to induce the apoptosis of those cells. In our experiments, however, Bcl-2 overexpression did consistently protect the lymphocytes from tumor-mediated killing. The protection from apoptosis was likely afforded by the ability of overexpressed Bcl-2 to defend Jurkat cells from cytochrome c release and the activation of the caspase cascade (Fig. 5). Although SK-RC-45 lowered Bcl-2 levels in cocultured T cells and Jurkat cells, we did not detect any cleavage products as reported by Gastman et al. (23). Whether this relates to differences in the Abs used in Western analysis or to a distinct mechanism is not clear.

Prior studies suggest that various cellular proteases cleave Bcl-2, Bcl-xl, Bid, Bax, and Bad, giving rise to truncated proteins with potent proapoptotic activity (22, 50). Bcl-2 specifically has been shown to undergo caspase-3-mediated cleavage after treatment of cells with apoptogenic stimuli, further facilitating apoptosis (51). In our experiments, however, SK-RC-45-mediated Bcl-2 degradation was largely caspase independent because inhibitors of caspase-3 and -9 were completely unable to reverse tumor-induced Bcl-2 disappearance, and the pan-caspase III inhibitor had only a minimal effect. So although caspase inhibitors can abrogate SK-RC-45-mediated T-cell apoptosis, they are unable to prevent SK-RC-45-induced Bcl-2 depletion, rendering T cells sensitive to other tumor-derived apoptogenic stimuli. In this regard, we are presently investigating the possibility that, like its parent compound, ceramide, tumor gangliosides may induce phosphatase activity (52) within the lymphocytes, which, by dephosphorylating Bcl-2, can promote the ubiquitination and subsequent proteosomal degradation of that antiapoptotic protein (53). The capacity of Bcl-2 overexpression to abrogate SK-RC-45-mediated apoptosis of T cells suggests that Bcl-2 inhibition is a mechanism by which tumors render lymphocytes sensitive to additional proapoptotic stimuli.

REFERENCES


The Bcl-2 Transgene Protects T Cells from Renal Cell Carcinoma-mediated Apoptosis

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