Induction and Regulation of Tumor Necrosis Factor-related Apoptosis-inducing Ligand/Apo-2 Ligand-mediated Apoptosis in Renal Cell Carcinoma

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ABSTRACT

The lack of effective therapy for disseminated renal cell carcinoma (RCC) has stimulated the search for novel treatments including immunotherapeutic strategies. However, poor therapeutic responses and marked toxicity associated with immunological agents has limited their use. The tumor necrosis factor family member tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/Apo-2 ligand induces apoptosis in a variety of tumor cell types, while having little cytotoxic activity against normal cells. In this study the activation and regulation of TRAIL-induced apoptosis and TRAIL receptor expression in human RCC cell lines and pathologic specimens was examined. TRAIL induced caspase-mediated apoptotic death of RCC cells with variable sensitivities among the cell lines tested. Compared with TRAIL-sensitive RCC cell lines (A-498, ACHN, and 769-P), the TRAIL-resistant RCC cell line (786-O) expressed lesser amounts of the death-inducing TRAIL receptors, and greater amounts of survivin, an inhibitor of apoptosis. Incubation of 786-O with actinomycin D increased the expression of the death-inducing TRAIL receptors and, concomitantly, decreased the intracellular levels of survivin, resulting in TRAIL-induced apoptotic death. The link between survivin and TRAIL regulation was confirmed when an increase in TRAIL resistance was observed upon overexpression of survivin in the TRAIL-sensitive, survivin-negative RCC line A-498. These findings, along with our observation that TRAIL receptors are expressed in RCC tumor tissue, suggest that TRAIL may be useful as a therapeutic agent for RCC and that survivin may partially regulate TRAIL-induced cell death.

INTRODUCTION

Apoptosis is an innate process in the life of complex organisms whereby unwanted cells are rapidly eliminated after controlled death. Apoptotic cell death plays a vital role during embryonic development, tissue remodeling, immune regulation, and tumor regression (1). Whereas there are multiple agents that can induce apoptosis, perhaps the best characterized are the death-inducing members of the TNF family of cytokines and their cognate receptors (2–4). For example, TNF and FasL function as inducers of apoptosis in many physiological events including autoimmune, activation-induced cell death, and immune privilege (5–8). TNF and FasL are also extremely efficient at killing a variety of tumor cells; however, they can cause significant damage to normal tissues resulting in life-threatening toxicities. TRAIL/Apo-2L is another TNF family member capable of inducing tumor cell apoptosis, and has received recently a great deal of attention because approximately two-thirds of the hematopoietic and nonhematopoietic tumor cell lines tested in vitro demonstrate sensitivity to recombinant, soluble forms of TRAIL (9–13). Additionally, and perhaps more importantly, TRAIL displays minimal cytotoxicity toward normal cells and tissues in vitro and in vivo, suggesting the development of TRAIL as a broad-spectrum, antitumor therapeutic molecule (9, 14–16).

Approximately 32,000 new cases of RCC are diagnosed yearly in the United States, and nearly 12,000 deaths are expected from RCC in 2002 (17). Metastatic RCC carries a median survival of 8 months, and almost 30% of RCC patients are diagnosed with advanced metastatic disease (17). Furthermore, RCC is highly resistant to chemotherapy, possibly through its association with the multidrug-resistant P-glycoprotein (18–21). Because of the ineffectiveness of chemotherapy against disseminated RCC, immunotherapy has been sought as an alternate treatment strategy for this disease. Agents such as IL-2, IFN-α, IFN-γ, tumor-infiltrating lymphocytes, and lymphokine-activated killer cells have been tested as potential therapies (17, 22–24). Despite the development of these immunotherapeutic strategies, overall response rates still remain poor, ranging from 10–30% with only limited durability of response and minimal improvement in survival time (22–24). Because of the potent apoptotic effects of TRAIL and the broad sensitivity of many tumor types to this agent, we evaluated its cytotoxic effect in established human RCC cell lines and investigated the mechanism(s) that regulate this effect. The results demonstrate that TRAIL induces apoptosis of RCC lines with variable sensitivity, which is associated with tumor cell-surface levels of the death-inducing TRAIL receptors and the intracellular levels of the IAP survivin. Furthermore, actinomycin D sensitized resistant cell lines to the cytotoxic effect of TRAIL by modulating both TRAIL receptor and survivin expression, and collectively these findings suggest that TRAIL should be investigated as a potential therapy for RCC.

MATERIALS AND METHODS

Materials. Reagents and sources used in this study were as follows: recombinant human TRAIL and anti-6X histidine mAb (R&D Systems, Minneapolis, MN); z-VAD-fmk (Enzyme Systems Products, Livermore, CA); anti-caspase-8 mAb (provided by Dr. Marcus Peter, University of Chicago, Chicago, IL); anti-PARP mAb (PharMingen, San Diego, CA); anti-survivin polyclonal antibody (25); MOPC-21, nonspecific IgG1 isotype control and MOPC-173, nonspecific IgG2a isotype control (Sigma, St. Louis, MO); z-VAD-fmk (Enzyme Systems Products, Livermore, CA); anti-PARP mAb (PharMingen, San Diego, CA); anti-survivin polyclonal antibody (25); MOPC-21, nonspecific IgG1 isotype control and MOPC-173, nonspecific IgG2a isotype control (Sigma, St. Louis, MO). The mAb against the four TRAIL receptors (M271, IgG2a anti-TRAIL-R1; M413, IgG1 anti-TRAIL-R2; M430, IgG1 anti-TRAIL-R3; and M444, IgG1 anti-TRAIL-R4) were produced at Immunex Corporation (Seattle, WA; Ref. 26).

Cell Lines. The human RCC cell lines, ACHN, A-498, 769-P, and 786-O, were obtained from American Type Tissue Culture (Rockville, MD). ACHN and A-498 were cultured in Eagle’s MEM supplemented with 10% FCS, 1% nonessential amino acids, 1 mm sodium pyruvate, and 1% streptomycin/penicillin solution. 769-P and 786-O were cultured in RPMI 1640 supple-

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4 The abbreviations used are: TNF, tumor necrosis factor; FasL, Fas ligand; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Apo-2L, Apo-2 ligand; RCC, renal cell carcinoma; IL-2, interleukin; mAb, monoclonal antibody; RT-PCR, renal proximal tubule epithelial cell; PI, propidium iodide; PARP, poly(ADP-ribose) polymerase; Ad-survivin, adenovirus encoding survivin; Ad-fgfl, adenovirus encoding β-galactosidase; IAP, inhibitor of apoptosis protein; hTRAIL, human tumor necrosis factor-related apoptosis-inducing ligand.
mented as above. Normal human RPTECs were obtained from Clonetics Corporation (San Diego, CA) and cultured as directed.

**Tumor Specimens.** Incisional and excisional biopsies from six patients with RCC were snap frozen and stored at −80°C. The University of Iowa Institutional Review Board approved the use of tumor specimens.

**In Vitro Killing with TRAIL.** Sensitivity to TRAIL was assayed by incubating the cells in 96-well plates (2 × 10^4 cells/well) with recombinant human TRAIL (at the indicated concentrations) and anti-6X histidine mAb (10 µg/ml) for 24 h. In some experiments, actinomycin D (20 ng/ml) or z-VAD-fmk (20 µM) was added to the culture medium immediately before the addition of TRAIL. Cell death was determined by crystal violet staining as described (27), with results presented as percentage of cell death: ([1 − (absorbance cells treated with TRAIL and anti-6X histidine mAb per absorbance cells with anti-6X histidine mAb)] × 100). The presence of the anti-6X histidine mAb during the assay had no effect on the viability of the target cells when compared with target cells cultured in medium alone.

**In Vitro Apoptosis Assay.** FITC-labeled annexin V was used to detect phosphatidylserine expression on early phase apoptotic cells by flow cytometry (28). RCC cells were incubated with TRAIL for 8 h, after which the cells were collected and resuspended in 100 µl of incubation buffer [10 mM HEPES/NaOH, (pH 7.4), 140 mM NaCl, and 5 mM CaCl_2] containing 20 µl of annexin V-FITC (Boehringer Mannheim) and 20 µl of PI (50 µg/ml) solution for 10 min. After adding an additional 400 µl of incubation buffer, cells were analyzed on a FACScan (Becton Dickinson, San Jose, CA).

**Flow Cytometry for TRAIL Receptor Expression.** Each RCC line and the normal RPTEC were incubated with the following unlabeled primary mAb for 1 h at 4°C: MOPC-21, MOPC-173, M271, M413, M430, or M444. After three washes, primary antibody binding was detected with a phycoerythrin-conjugated, Fc-specific, goat antimouse F(ab')2 (Jackson Immunoresearch, Rockyford, IL). Equal amounts of protein were separated by SDS-PAGE, and the membrane was incubated with an antimouse-horseradish peroxidase-labeled antibody (diluted according to manufacturer’s instructions) for 1 h. After washing, the membrane was incubated with an antimonouse-horseradish peroxidase-conjugated antibody (diluted 1:1000; Amersham, Arlington Heights, IL) for 1 h. After several washes, the blots were developed with chemiluminescence according to the manufacturer’s protocol (Renressence chemiluminescence reagent; DuPont NEN, Boston, MA).

**Western Blotting.** Cells from each RCC line were lysed in PBS containing 1% NP40, 0.35 mg/ml phenylmethylsulfonyl fluoride, 9.5 µg/ml leupeptin, and 13.7 µg/ml pepstatin A. The lysed cells were centrifuged at 14,000 × g to remove cellular debris. Protein concentrations of the extracts were determined by the colorimetric bichinonic acid analysis (Pierce Chemical Company, Rockford, IL). Equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose membrane (Novex, San Diego, CA), and blocked with 5% nonfat dry milk in PBS-Tween 20 (0.05% v/v) overnight. The membrane was incubated with the anticaspase-8, - PARP, or - survivin antibodies (diluted according to manufacturer’s instructions) for 1 h. After washing, the membrane was incubated with an antimouse-horseradish peroxidase-conjugated antibody (diluted 1:1000; Amersham, Arlington Heights, IL) for 1 h. After several washes, the blots were developed with chemiluminescence according to the manufacturer’s protocol (Renressence chemiluminescence reagent; DuPont NEN, Boston, MA). Equal amounts of protein were separated by SDS-PAGE for Western blot analysis of caspase-8 activation and PARP cleavage. Caspase-8 activation and PARP cleavage were detected within 2 h after TRAIL addition and peaked by 4 h (Fig. 2A). However, by 24 h, the levels of the active p18 subunit of caspase-8 and M1, 85,000 fragment of PARP had dropped below the level of detection because of extensive apoptotic destruction. To additionally demonstrate the importance of caspase activation in the TRAIL-induced apoptosis of A-498, the caspase inhibitor z-VAD-fmk [carbobenzyloxy-Val-Ala-Asp (OMe) fluoromethyl ketone] was added to the culture medium before the addition of TRAIL-z-VAD-fmk completely inhibited TRAIL-mediated A-498 cell death, whereas equal concentrations of the peptide vehicle (DMSO) did not (Fig. 2B). Finally, TRAIL-induced apoptosis of A-498 cells was measured by annexin V binding (Fig. 2C). These results demonstrate that the TRAIL-sensitive RCC lines are killed by an apoptotic mechanism.

**TRAIL Receptor Expression in RCC.** TRAIL interacts with four distinct receptors: DR4 (37), DR5/TRAIL-R2 (34, 38, 39), TRID/DecR1/TRAIL-R3 (37–40), and TRAIL-R4/DecR2 (11, 41; hereafter referred to as TRAIL-R1, -R2, -R3, and -R4, respectively). Both TRAIL-R1 and TRAIL-R2 contain a cytoplasmic death domain, and cross-linking by TRAIL- or receptor-specific mAb activates the apoptosis-signaling pathway in sensitive cells (34, 37–39). In contrast, neither TRAIL-R3 (which is glycosylphosphatidyl inositol linked) nor TRAIL-R4 (which is a type I membrane protein) contains a complete cytoplasmic death domain, and neither can mediate apoptosis on ligation (11, 38–41). Because they bind to TRAIL without directly signaling for cell death, it was proposed that TRAIL-R3 and TRAIL-R4 may serve as protective receptors, acting either as antagonic receptors (38–41) or as receptors transducing an antiapoptotic signal (11). Therefore, the differential expression of the TRAIL receptors may determine whether a cell is resistant or sensitive to TRAIL-induced apoptosis (38, 39, 41). Thus, TRAIL receptor expression on the four RCC lines and the RPTEC was analyzed by flow cytometry. The data demonstrate TRAIL-R1 and -R2 is expressed on all four of the RCC cell lines, albeit at lower amounts on 769-P.
Mcleavage was determined by Western blot analysis. Caspase-8 activation produces an 85,000 occurs during apoptosis. For comparison, lysates from untreated A498 cells were also examined. B, inhibition of hTRAIL-induced apoptosis in A-498 cells by z-VAD-fmk. Microtiter plates (96-well flat-well) were seeded with 2 × 10^3 cells/well and allowed to adhere for at least 6 h. z-VAD-fmk (20 μM), DMSO, or medium was added to each well immediately before adding hTRAIL at the indicated concentrations and anti-6X histidine mAb (10 μg/ml). Cell viability was determined after 24 h by crystal violet staining. Each value represents the mean of three wells. For clarity, SD bars were omitted from the graph, but were <5% for all data points. Experiments were performed at least three separate times with similar results. C, annexin V staining of A-498. Phosphatidylserine (PS) externalization on RCC after hTRAIL treatment. A-498 tumor cells were cultered for 8 h in medium alone or in the presence of hTRAIL (20 or 50 ng/ml) and anti-6X histidine mAb (10 μg/ml). Cells were then stained with FITC-annexin V and PI and analyzed by flow cytometry. The percentage of FITC-annexin V^+PI^+ tumor cells is indicated for each condition. Histograms represent 10^4 gated tumor cells.

Fig. 2. TRAIL induces apoptotic death of the RCC line A-498. A, kinetics of caspase-8 and PARP cleavage after hTRAIL treatment. Plates (24-well) containing 5 × 10^3 A-498 cells were treated with hTRAIL (100 ng/ml) and anti-6X histidine mAb (10 μg/ml), cell lysates were prepared at the indicated times after infection, and caspase-8 and PARP cleavage was determined by Western blot analysis. Caspase-8 activation produces an 85,000 active subunit from the zymogen. PARP cleavage was determined by Western blot analysis, and all of the samples were found to express TRAIL-R1 and -R2 (Table 1).

When treated with TRAIL in the presence of actinomycin D, 786-O cells were readily killed by TRAIL (Fig. 4A). By comparison, the addition of actinomycin D to normal RPTEC only slightly increased sensitivity at the highest TRAIL concentrations. Additionally, caspase-8 activation was observed in 786-O when treated with TRAIL and actinomycin D, but not with TRAIL alone (Fig. 4B). These results demonstrate that 786-O contains the required components to undergo TRAIL-induced apoptosis, but the apoptotic machinery is inhibited by an actinomycin D-sensitive mechanism.

Flow cytometric analysis revealed that 786-O expressed low levels of TRAIL-R1 and -R2 (Fig. 3). Therefore, we hypothesized that the observed increase in TRAIL sensitivity of actinomycin D-treated 786-O cells was potentially the consequence of an increase in TRAIL death receptor expression. After actinomycin D treatment, an increase in cell surface levels of TRAIL-R1 and -R2 on 786-O was observed by flow cytometry (Fig. 5). However, TRAIL-R3 and -R4 levels were not altered by actinomycin D (data not shown). Similarly, actinomycin D-treatment RPTECs demonstrated a slight but detectable increase in TRAIL-R1 and -R2 levels when compared with untreated cells. The observed modulation of TRAIL-R1 and -R2 levels by actinomycin D provides a possible explanation for the conversion of 786-O from TRAIL-resistant to TRAIL-sensitive, but the fact that TRAIL-R1 and -R2 can be detected on the surface of untreated cells suggests that there are potentially other regulatory mechanisms present that inhibit apoptosis after TRAIL ligation.

Intracellular Levels of the IAP Member Survivin in RCC Correlate with TRAIL Sensitivity. Recent reports demonstrate that there are several intracellular proteins capable of inhibiting death receptor-mediated apoptosis when present at sufficient levels (43–45). One such molecule is survivin, a recently identified member of the IAP family that is selectively expressed in tissues during development but not in terminally differentiated mature tissue (46) and has been shown to be associated with tumors of poor prognosis (47, 48). Moreover, survivin blocks apoptosis by direct inhibition of downstream effector caspases, and when overexpressed in cancer cells leads to aberrant proliferation through mitosis (49–51). Survivin has also been detected in pediatric renal tumors, and its presence is a marker of worse prognosis (52). Thus, survivin expression was assessed in the four RCC lines by immunoblotting. As demonstrated in Fig. 6A, the TRAIL-resistant RCC line 786-O expressed the highest levels of survivin protein. By comparison, survivin levels in the TRAIL-sensitive lines ACHN, A-498, and 769-P were dramatically lower or absent. However, RNase protection assay analysis found survivin mRNA present in all four of the RCC lines, with 786-O containing the highest amount (data not shown).

We proposed that intracellular regulators of apoptosis, like survivin, play a pivotal role in determining cell sensitivity to apoptosis inducing ligands. If survivin has a high turnover rate within the cell, then the intracellular levels should decrease over time when the cells are exposed to actinomycin D. The survivin-expressing, TRAIL-resistant RCC line 786-O was incubated with actinomycin D, after which survivin levels were again determined by immunoblotting. As predicted, survivin levels were found to decrease with actinomycin D exposure (Fig. 6B). These results suggest that the expression of apoptosis-inhibiting molecules like survivin, coupled with low expression levels of the death-inducing TRAIL receptors, influence RCC susceptibility to TRAIL-mediated apoptosis.

To directly investigate the link between survivin and TRAIL-induced apoptosis in RCC, the TRAIL-sensitive, survivin-deficient cell line A-498 was infected with a recombinant Ad-survivin or Ad-βgal. Survivin levels were determined by immunoblotting wildtype A-498 cells and A498 cells infected with either Ad-βgal or Ad-survivin (Fig. 6C), with survivin only expressed in the A-498 cells.
infected with Ad-survivin. Simultaneously, the transfectants were tested for TRAIL sensitivity, and the Ad-survivin-infected A-498 cells proved to be remarkably more resistant to TRAIL-induced apoptosis (Fig. 6D). Whereas these results directly link survivin expression to TRAIL-induced apoptosis in RCC, it is likely that survivin is just one of many molecules that regulate TRAIL-receptor mediated apoptosis.

**DISCUSSION**

The lack of effective therapy for disseminated RCC has led to the investigation and development of various immunological treatment strategies (17, 22–24). IFN-α is one of the most widely used biological response modifiers for RCC. A comparative study examining the use of IFN-α alone or in combination with IL-2 in the treatment of RCC revealed overall response rates of 6.5 and 18.6%, respectively (22). However, there was no difference in survival times between the groups, and there was significantly greater toxicity when both IFN-α and IL-2 were used. These poor clinical response rates, along with the associated toxicities, have limited the use of such immune mediators in the treatment of RCC, making the identification and development of other, less toxic, biological molecules a critical goal. The TNF superfamily is a group of cytokines that exert a variety of effects on different cells, such as cellular activation, proliferation, and death (2, 3). In this report the tumoricidal activity of the TNF superfamily member, TRAIL, was examined on several human RCC cell lines and tissues, and normal RPTECs. Consistent with previous studies, we found that even within a specific tumor type sensitivity to TRAIL can be variable (12, 53). The RCC lines ACHN and A-498 displayed the greatest sensitivity to the cytotoxic effects of TRAIL, whereas 769-P was moderately sensitive, and 786-O and normal RPTEC were TRAIL resistant. We were then able to extend the observations reported previously (15, 31–33) to examine potential mechanisms that regulate TRAIL sensitivity in the RCC lines. Our investigation revealed that the TRAIL-resistant line 786-O, unlike RPTEC, could be converted to TRAIL-sensitive by blocking transcription with actinomycin D, demonstrating that this line was not fundamentally defective in the apoptotic signaling machinery. Instead, these cells expressed low levels of the death-inducing TRAIL receptors and high levels of the apoptosis inhibitor survivin, indicating that multiple factors within the cell and at the cell surface can provide protection against the cytotoxic effects of TRAIL.

Monomeric human TRAIL consists of 281 amino acids, and like most of the other members of the TNF family, is a type II membrane protein (9). Analysis of the extracellular domain of TRAIL revealed it to be most homologous to that of FasL (28% amino acid identity), Table 1

<table>
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<th>Patient</th>
<th>Age/sex</th>
<th>RCC type</th>
<th>Stage-grade</th>
<th>Survivin</th>
<th>TRAIL-R1 mRNA</th>
<th>TRAIL-R1 Protein</th>
<th>TRAIL-R2 mRNA</th>
<th>TRAIL-R2 Protein</th>
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<td>T2NM0M0</td>
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<td>+</td>
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<tr>
<td>C</td>
<td>84/M</td>
<td>Papillary</td>
<td>Grade I</td>
<td>T2NM0</td>
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<tr>
<td>D</td>
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<td>Papillary</td>
<td>Grade II</td>
<td>T2NM0</td>
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a NED, no evidence of disease; ESRD, end-stage renal disease.

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Fig. 3. Surface analysis of RCC and RPTEC for TRAIL receptors. Flow cytometric analysis of TRAIL-R1, -R2, -R3, and -R4 expression on ACHN, A-498, 769-P, 786-O, and RPTEC. Solid line histograms represent staining by M271 (anti-TRAIL-R1 mAb), M413 (anti-TRAIL-R2 mAb), M430 (anti-TRAIL-R3 mAb), or M444 (anti-TRAIL-R4 mAb), and dotted line histograms represent staining with isotype control mAb. Histograms represent 10⁴ gated tumor cells.
Figure 4. Actinomycin D makes the resistant RCC line 786-O sensitive to hTRAIL-induced apoptosis with caspase activation. A. 96-well flat-bottomed microtiter plates were seeded with 786-O or RPTEC (2 × 10^4 cells/well) and allowed to adhere for at least 6 h. Actinomycin D (20 ng/ml) or medium was added to each well immediately before adding hTRAIL at the indicated concentrations and anti-6X histidine mAb (10 μg/ml). Cell viability was then determined after 24 h by crystal violet staining. Each value represents the mean of three wells. For clarity, SD bars were omitted from the graph, but were <5% for all data points. Experiments were performed at least three separate times with similar results. B. 24-well plates containing 5 × 10^5 786-O cells were treated with hTRAIL (100 ng/ml) and anti-6X histidine mAb (10 μg/ml) for the indicated times with or without actinomycin D (20 ng/ml), after which cell lysates were prepared and caspase-8 activation was determined by Western blot analysis.

Figure 5. Increase in TRAIL-R1 and -R2 expression on 786-O cells occurs with actinomycin D treatment. Surface levels of TRAIL-R1 and -R2 were examined on 786-O and RPTEC cells after 24 h culture in medium alone or medium containing actinomycin D (20 ng/ml). Thick solid line histograms represent staining by M271 (anti-TRAIL-R1 mAb) or M413 (anti-TRAIL-R2 mAb) on actinomycin D-treated cells; thin solid lines represent staining by the same mAb without actinomycin D, and dotted line histograms represent staining with isotype control mAb. Histograms represent 10^5 gated tumor cells.

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followed by TNF-α (23%), lymphotoxin α (23%), and lymphotoxin β (22%; Ref. 9). Whereas the homology of TRAIL to other TNF family members may be considered low, examination of the crystal structure of monomeric TRAIL found it to be quite similar to that of TNF-α, TNF-β, and CD40 ligand (54). TRAIL monomers contain two antiparallel β-pleated sheets that form a β sandwich core framework, and these monomers interact with other TRAIL monomers in a head-to-tail fashion to form a bell-shaped trimer (54). This oligomerization enhances the activity of TRAIL, because studies with recombinant soluble TRAIL found that the most biologically active form was multimeric, or cross-linked, rather than monomeric (9).

Early studies characterizing TRAIL also identified that it induced apoptotic cell death only in tumorogenic or transformed cells and not in normal cells in vitro (9, 10). Moreover, mRNA for TRAIL was detected in many tissues, including peripheral blood leukocytes, spleen, thymus, prostate, ovary, small intestine, colon, and placenta, but not brain, liver, or testis (9). This distribution was somewhat unusual in that the mRNA expression of other members of the TNF family is tightly regulated and only transiently expressed on activated cells (4, 55). This broad expression pattern also suggested that TRAIL was not cytotoxic to most tissues in vivo, and TRAIL-induced apoptosis was likely regulated either through restricted receptor expression or only occurred under specific circumstances. In results presented herein, TRAIL was highly cytotoxic to two of the human RCC lines but not normal RPTECs, which is consistent with previous reports demonstrating the tumor-specific activity of TRAIL (9, 10, 14, 15, 34). Moreover, TRAIL induced apoptotic death of the sensitive RCC lines, as evidenced by caspase activation, intracellular protein cleavage, and annexin V binding. Morphological changes, such as membrane blebbing and release of apoptotic bodies, were also observed using light microscopy (data not shown).

These events, indicative of apoptosis, were not observed in the resistant RCC line 786-O or normal RPTEC, prompting their evaluation for the presence of extracellular and/or intracellular regulation. In the TRAIL/TRAIL receptor system, the four TRAIL receptors bind TRAIL with comparable affinities (11, 40). TRAIL-R3 and -R4 are primarily expressed in normal tissues and not in tumor cells (38, 39, 41), leading to the initial conclusion that expression of either or both of these receptors provided resistance to TRAIL-induced apoptosis. However, these initial reports investigating the distribution of the different TRAIL receptors did so only at the mRNA level (37–39, 41), and it was unknown whether physiological expression levels of TRAIL-R3 and -R4 were sufficient to interfere with TRAIL-induced apoptosis as was demonstrated in overexpression experiments (37, 38, 41). With the development of TRAIL receptor-specific mAb, analysis of a variety of human tumor cells lines demonstrated no correlation between cell surface TRAIL-R3 and -R4 expression, and the level of sensitivity to TRAIL-induced apoptosis (26, 53). When analyzing TRAIL receptor expression on the four RCC lines and the normal RPTEC, it was found that each line differentially expressed both TRAIL-R1 and -R2. Specifically, the TRAIL-sensitive lines
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Several intracellular inhibitors of apoptosis are identified that regulate TRAIL receptor-mediated cell death. For example, overexpression of the viral caspase inhibitors CrmA or p35 can block TRAIL-induced apoptosis (43). Likewise, several studies have reported a correlation between high expression of FADD-like IL-β-converting enzyme-inhibitory protein (FLIP) and TRAIL-resistance (12, 56, 57). Here, we observed high levels of the IAP family member survivin in the TRAIL-resistant RCC line 786-O, whereas the TRAIL-sensitive lines A-498, ACHN, and 769-P had much lower but detectable amounts of survivin. This is not surprising, because immortalized tumor cell lines are usually developed from highly malignant tumors of poor prognosis. Moreover, overexpression of survivin in A-498 cells from a recombinant adenovirus resulted in a complete protection against low concentrations of TRAIL but only partial protection against high TRAIL doses. Recent observations have demonstrated that survivin inhibits apoptosis induced by a variety of agents by interfering with caspase activation, namely caspase-3, -7, and -9 (51). Cleavage of caspases occurs during TRAIL-induced apoptosis, suggesting that survivin may contribute to the regulation of this event. Furthermore, A-498 expresses high amounts of TRAIL-R1 and -R2 (Fig. 3), so it is possible that level of survivin produced from the adenoviral transgene was not high enough to fully neutralize the massive apoptotic signal generated at the high TRAIL concentrations. Survivin is normally expressed during fetal development, and is rarely found in normal adult cells and tissues (46–48). However, expression of survivin has been noted in a variety of human tumors, including pediatric renal tumors, suggesting that increased levels of survivin contribute to tumorigenesis (46–48, 52, 58). In contrast, survivin was not detected in the normal RPTECs examined in this study, as determined by immunoblot analysis (data not shown). This would suggest that other mechanisms are present in normal RPTECs that function to protect these cells from the cytotoxic effects of TRAIL. Moreover, survivin was not detected in the six RCC tumor specimens surveyed (Table 1). The absence of survivin in these clinical tumors is of interest, because all of these patients are in remission without indication of active disease. As with many other tumor types, the absence of survivin in RCC may be a favorable prognostic indicator.

There are potentially multiple regulatory components in the TRAIL/TRAIL receptor system that influences the sensitivity of the cell to TRAIL-induced apoptosis. It is apparent from our findings that both TRAIL receptor expression and the presence of intracellular inhibitors of apoptosis, like survivin, are critical determinants for RCC sensitivity to TRAIL. When used alone, TRAIL appears to be a potent cytotoxic agent in several of the RCC lines tested, whereas in the most TRAIL-resistant RCC line (786-O), TRAIL resistance was overcome by adjuvant treatment with actinomycin D. These unique in vitro observations may have important clinical implications for the application of TRAIL in the treatment of renal cancer.

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Fig. 6. Susceptibility to TRAIL-induced apoptosis in RCC lines is related to levels of survivin. A. Levels of survivin protein in the four human RCC lines were measured in equivalent cellular lysate amounts by Western blot analysis. B. Survivin levels decreased over time in the TRAIL-resistant RCC line 786-O when treated with actinomycin D. Six-well plates containing 5 × 10⁴ cells were incubated with actinomycin D (20 ng/ml) for the indicated times. Cellular lysates were then separated by SDS-PAGE, transferred to nitrocellulose, and survivin expression was determined by Western blot analysis. C. Expression of survivin in A-498 cells after infection with Ad5-survivin. A-498 cells (5 × 10⁵) were added to T-25 tissue culture flasks and allowed to adhere for at least 6 h before infecting with either Ad5-control or Ad5-survivin (50 plaque-forming units/cell). D. After an additional 12-h incubation, cell lysates were prepared, and survivin levels were determined by Western blot analysis. To demonstrate equivalent protein loading, lysates were also analyzed for β-actin levels. D. Survivin-expressing A-498 cells are resistant to hTRAIL-induced death. A-498 cells infected as in C were added to 96-well flat-bottomed microtiter plates (2 × 10⁴ cells/well) and allowed to adhere for at least 6 h before treating with hTRAIL and anti-6XHis histidine mAb (10 μg/ml). Cell viability was determined after 24 h by crystal violet staining. Each value represents the mean of three wells. For clarity, SD bars were omitted from the graph but were <5% for all data points. The results in D are representative of three independent experiments.

ACHN and A-498 expressed the highest levels of TRAIL-R1 and -R2. 769-P, which was moderately sensitive to TRAIL, also expressed both TRAIL-R1 and -R2, but at a lower level. Finally, the resistant RCC line 786-O and normal RPTEC had the lowest amounts of TRAIL-R1 and -R2 on their surface. In contrast, none of the cells expressed TRAIL-R3, and only the two most sensitive lines (ACHN and A-498) had detectable levels of TRAIL-R4. Whereas these results did not indicate that the expression of TRAIL-R3 and -R4 conferred resistance to any of the lines, they did suggest that the lower levels of TRAIL-R1 and -R2 on 786-O and RPTEC correlate with TRAIL resistance.

It is of note that the conversion of the TRAIL-resistant line 786-O to TRAIL-sensitive by treatment with actinomycin D was marked by an increase in surface expression of TRAIL-R1 and -R2, suggesting that TRAIL-R1 and/or -R2 levels must reach a certain level before a strong enough signal can be generated to initiate the apoptotic pathway. The exact mechanism of this intriguing observation is unknown, but increased receptor expression may be because of a change in the rate of receptor turnover at the cell surface. Hypothetically, the protein(s) responsible for cleaving TRAIL-R1 and/or -R2 from the cell membrane or for receptor turnover are actinomycin D sensitive, allowing already translated TRAIL-receptor protein to accumulate at the cell surface. Whatever the mechanism, we have also observed similar increases in TRAIL-receptor expression in neuroblastoma cell lines after treatment with etoposide. This novel observation may have important clinical implications for using immunotherapeutic agents as adjuvants to chemotherapeutic drugs.

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