Combination of Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) and Actinomycin D Induces Apoptosis Even in TRAIL-resistant Human Pancreatic Cancer Cells

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a novel member of the tumor necrosis factor superfamily of cytokines that induces cell death by apoptosis. TRAIL has been shown to be effective in almost two-thirds of solid tumors tested thus far, but its effect on pancreatic cancer cells is unknown. We tested the effect of TRAIL on seven human pancreatic cancer cell lines (HPAF, Panc1, Miapaca2, Bxpc3, Panc89, SW979, and Aspc1) in vitro. Of these cell lines, all but Aspc1 showed a significant dose-dependent increase in apoptosis. The apoptotic rate, as detected by a terminal deoxynucleotidyl transferase-mediated nick end labeling assay, was highest in Bxpc3 (71.5%), followed by HPAF (38.0%), Miapaca2 (24.9%), Panc1 (16.1%), Panc89 (15.8%), SW979 (13.9%), and Aspc1 (5.2%). Multiple treatments were more effective than a single treatment and caused a sustained and profound cell death in all but Aspc1 cells. There was no correlation between the effect of TRAIL and the differentiation grade of the cell lines, p53 mutation, or bcl-2 or bax expression. The resistance of Aspc1 cells to TRAIL was not related to the lack of TRAIL receptors. The combination of actinomycin D and TRAIL induced an almost complete lysis of Aspc1 cells, whereas actinomycin D alone had no effect on cell survival but inhibited the expression of the Flice inhibitory protein, which is assumed to play a role in the apoptotic pathway of TRAIL. Thus, the combination of actinomycin D and TRAIL appears to be a promising approach for the therapy of pancreatic cancers resistant to TRAIL.

INTRODUCTION

Cytokines are a family of proteins that regulate cellular proliferation and differentiation by binding to their specific receptors on target cells. They are grouped into at least three subfamilies: (a) cysteine knot factors; (b) TNFs; and (c) helical cytokines. The TNF family includes TNF-α, FasL, lympho-toxin, CD30 ligand, 4-1BB ligand, CD40 ligand, CD27 ligand, and TRAIL (1). Most TNF family cytokines are expressed as a type II transmembrane protein with its NH2 terminus in the cytoplasm and its COOH-terminal region extending into the extracellular space. The COOH-terminal extracellular domain is processed proteolytically to form a soluble homotrimERIC molecule (2). TRAIL (also called APO2 ligand) is a newly discovered TNF superfamily member initially cloned from human heart and lymphocyte cDNA libraries (3). With a predicted molecular weight of 32,000, human TRAIL is 281 aa residues long, with a 17-aa residue cytoplasmic tail, a 21-aa residue transmembrane segment, and a 243-aa residue extracellular region (2, 3). Human TRAIL is 65% identical to mouse TRAIL at the aa sequence level across the entire molecule, and there is a complete species cross-reactivity (3). Although TRAIL is known to be expressed by lymphocytes, many tissues seem to express the ligand, and its broad expression pattern suggests an intriguing function for the molecule (3).

TNF family members induce cell death by apoptosis; hence, their receptors are also called “DRs.” T-cell cytotoxicity is mediated primarily by FasL and TRAIL (4, 5). Their apoptotic properties have prompted investigation of the use of certain TNF family members in treating melanomas; mammary, colorectal, ovarian, cervical, bladder, and renal cancers; and hematological and some mesenchymal malignancies (4, 6–11). In this aspect, TRAIL has gained a central role because it also induces apoptosis in FasL-resistant tumor cells (4). Further-

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3 The abbreviations used are: TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; FLIP, Flice inhibitory protein; aa, amino acid(s); FasL, Fas ligand; RT-PCR, reverse transcription-PCR; PBS-T, PBS-Tween 20; Ab, antibody; DR, death receptor; DR, decay receptor.
more, contrary to FasL, TRAIL is not toxic to normal cells (6), although a new report indicates toxicity in normal human hepatocytes (12), and it does not have a graft-versus-host disease effect but mediates a favorable graft-versus-tumor effect (6).

Pancreatic cancer is the fifth leading cause of cancer deaths in the United States, killing about 28,000 people every year in the United States alone (13). The etiology of the diseases is still obscure, and early diagnosis and treatment remain disappointing. Consequently, it was of interest to examine the effect of TRAIL on pancreatic cancer cells. Although Fas and FasL have been shown to be expressed by human pancreatic cancer cells, they have been resistant to Fas-mediated apoptosis (14). Studies by Raitano et al. (15) have shown that TNF in combination with INF-γ inhibited the growth of some pancreatic cancer lines. However, to our knowledge, the effect of TRAIL on pancreatic cancer has not been tested. We examined the effect of TRAIL on seven pancreatic cancer lines in vitro and found marked apoptosis in six of these cell lines.

MATERIALS AND METHODS

Reagents and Abs. Recombinant human TRAIL (leucine-zipper construct; Ref. 8) was provided by the Immunix Corp. (Seattle, WA). Actinomycin D was purchased from Sigma (St. Louis, MO). The Abs for the four TRAIL receptors were provided by the Immunix Corp. Abs used for immunohistochemistry are summarized in Table 1.

Cell Lines. Human pancreatic cancer cell lines Panc1, Aspc1, MiaPaca2, and Bxpc3 were obtained from American Type Culture Collection; Panc89 and SW979 were obtained from Dr. S. K. Batra (University of Omaha, Nebraska, NE); and HPAF was obtained from Dr. M. A. Hollingsworth (The Eppley Institute, University of Nebraska, Omaha, NE). Panc1 and HPAF cells were cultured in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD), and the remaining cells were cultured in DMEM (Life Technologies, Inc.), and each was supplemented with 10% fetal bovine serum (Summit, CO), 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.) in a humidified atmosphere of 5% CO₂ in air.

Treatment. To assess the optimal concentration of TRAIL, in a pilot study 4 × 10⁴ cells from each cell line were seeded in 24-well plates. After 36–48 h of incubation at 37°C, the medium was changed, and TRAIL was added to the regular culture medium (1 ml/well) without fetal bovine serum at concentrations of 0.1, 1, 3.13, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 ng/ml. Aspc1 cells were also treated with 1600 ng/ml TRAIL. Control cells were cultured in the absence of TRAIL. After 24 h, the cells were washed with PBS, and the cells that were still attached were considered to be viable cells and counted by a hemocytometer. The IC₅₀ of TRAIL was determined by a 50% decrease of the viable cell number compared with untreated cells by a given concentration.

For a multiple treatment of TRAIL, 4 × 10⁴ cells from each cell line were cultured per well in a 6-well plate in regular culture medium (3 ml/well) without fetal bovine serum and treated with TRAIL at a dose of 200 ng/ml eight times, each time for 3 days. The cell number of each group was determined at the end of each treatment.

We also examined the effect of actinomycin D alone or in combination with TRAIL on Aspc1, HPAF, and Bxpc3 cells. Cells of each cell line (4 × 10⁴) were cultured in a 6-well plate. After 36 h of incubation, the cells were treated with TRAIL (200 ng/ml) and actinomycin D (0.1 or 1 μg/ml). After the treatment for 24 h, viable cells were counted. The effect of actinomycin D alone at doses of 0.1 or 1 μg/ml on Aspc1, HPAF, and Bxpc3 was also examined similarly. Aspc1, HPAF, and Bxpc3 were chosen because of their differing sensitivities to TRAIL.

TUNEL Assay. TUNEL assay was performed to determine the percentage of cells undergoing apoptosis after treatment with TRAIL or treatment with the combination of TRAIL and the protein synthesis inhibitor actinomycin D. Briefly, 1 × 10⁶ cells from each cancer cell line were seeded in T75 flasks and cultured for 2 days at 37°C before TRAIL was added at a concentration of 200 ng/ml. In the combined treatment, TRAIL was given at a dose of 200 ng/ml, and actinomycin D was given at a dose of 0.1 or 1 μg/ml. After 24 h of incubation, the cells were collected and fixed with 1% paraformaldehyde in PBS for 20 min on ice. After washing with PBS, the cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min on ice. After washing, the cells were incubated with FITC-conjugated dUTP in the presence of terminal deoxynucleotidyl transferase enzyme solution for 1 h at 37°C using the Apoptosis Detection System (Promega, Madison, WI). After incubation, cells were washed twice with 0.1% Triton X-100 in PBS containing 5 mg/ml BSA and then incubated with 5 μg/ml propidium iodide solution in PBS containing 250 μg of Dnase-free RNase A (Sigma) for 30 min at room temperature. Ten thousand cells were assessed by two-color FACScan analysis (Becton Dickinson, San Jose, CA).

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Table 1  Antibodies used for immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Pretreatment</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>P53 (Pab240)</td>
<td>Microwave heat</td>
<td>1:100</td>
<td>Biogenex (San Ramon, CA)</td>
</tr>
<tr>
<td>DU-PAN 2</td>
<td>None</td>
<td>1:10</td>
<td>Dr. Colcher (Omaha, NE)</td>
</tr>
<tr>
<td>CA 19-9</td>
<td>None</td>
<td>1:10</td>
<td>Wistar Institute (Philadelphia, PA)</td>
</tr>
<tr>
<td>B72.3</td>
<td>None</td>
<td>1:50</td>
<td>NIH (Bethesda, MD)</td>
</tr>
<tr>
<td>TGF-α*</td>
<td>0.05% saponin</td>
<td>1:20</td>
<td>Oncogene Research Products (Cambridge, MA)</td>
</tr>
<tr>
<td>EGFR</td>
<td>0.05% saponin</td>
<td>1:40</td>
<td>Sigma (St Louis, MO)</td>
</tr>
<tr>
<td>PI6</td>
<td>Microwave heat</td>
<td>1:40</td>
<td>Oncogene Research Products (Cambridge, MA)</td>
</tr>
<tr>
<td>Bel-2</td>
<td>Microwave heat</td>
<td>1:100</td>
<td>Oncogene Research Products (Cambridge, MA)</td>
</tr>
<tr>
<td>Bax</td>
<td>Microwave heat</td>
<td>1:20</td>
<td>Oncogene Research Products (Cambridge, MA)</td>
</tr>
<tr>
<td>Ki 67</td>
<td>Microwave heat</td>
<td>Ready to use</td>
<td>Biogenex (San Ramon, CA)</td>
</tr>
</tbody>
</table>

*TGF-α, transforming growth factor α; EGFR, epidermal growth factor receptor.
FITC-propidium iodide double-positive cells were considered apoptotic.

**DNA Fragmentation Analysis.** Induction of apoptosis was also determined by the internucleosomal DNA fragmentation method as described previously (16). Briefly, purified DNA was analyzed by electrophoresis on a 1% agarose gel at 25 V for 16 h.

**RT-PCR Analysis.** The expression of the mRNA for TRAIL receptors DR4, DR5, DcR1, and DcR2 in all seven cell lines was examined. Total RNA was purified using the guanidine thiocyanate acid phenol method (17). Briefly, 1 μg of total RNA was mixed with 100 ng of random hexamer primers, and the solution was heated at 72°C for 5 min and then placed on ice. Deoxynucleotide triphosphates (10 mM each), reverse transcriptase, Rnasin, 10 mM PCR buffer, 25 mM MgCl2, and water were added according to the manufacturer's instructions (Perkin-Elmer, Cy- press, CA). The mixture was incubated at 42°C for 60 min. cDNA synthesis reaction was terminated by heating at 95°C, and the mixture was stored at −70°C. Primer sequences for all four TRAIL receptors and the PCR protocol used in this study were published previously (18). Glyceraldehyde-3-phosphate dehydrogenase primers were used for the internal control (19). Primers for RT-PCR were synthesized by the UNMC Eppley Molecular Biology Core Laboratory (Omaha, NE). The sequences were as follows: (a) DR4, 5′-CGATGGCTGTCGAGCTCGACAGC-3′ (sense) and 5′-GGACAGGGCAGGCTGAGGACAT-3′ (antisense); (b) DR5, 5′-GGGAGGGCTCTATGAGGAAAGTTG-3′ (sense) and 5′-GGCAGTGCTCTCCACGCTTCTC-3′ (antisense); (c) DcR1, 5′-GGTGGTTTGGAAAGAGCTTCATGCT-3′ (sense) and 5′-GCAGGGCCTTCTGCTGCTG-3′ (antisense); (d) DcR2, 5′-GGCAGGGCCTTCTGCTGCTG-3′ (antisense); (e) glyceraldehyde-3-phosphate dehydrogenase, 5′-CGGATTGGTCTCATATTGG-3′ (sense) and 5′-TCCTGGAGATGTTGATG-3′ (antisense).

PCR products were analyzed by electrophoresis on 1.8% agarose gels.

**Immunocytochemistry.** Cells (5 x 10⁶) from each cell line were pelleted, fixed in 10% buffered formalin for 24 h, embedded in paraffin, cut in serial sections, and processed for immunocytochemistry by the Avidin Biotin Complex method (20). The cells were also grown on a Lab-TEK chamber slide (Nalge Nunc International, Naperville, IL), fixed with acetone, and processed for immunohistochemistry.

**Western Blot.** The cells were lysed in PBS containing 1% NP40 and protease inhibitor mixture (Boehringer Mannheim, Mannheim, Germany). The lysates were centrifuged at 14,000 x g to remove the debris, and the protein concentration was determined by using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein were separated by SDS-PAGE, transferred to a nitrocellulose membrane (Bio-Rad), and blocked with 5% NFDM in PBS-T overnight at 4°C. After washing with PBS-T, the membrane was incubated with TRAIL receptor monoclonal Abs or FLIP antisera (diluted 1:1,000) for 1 h at room temperature. After washing with PBS-T, the membrane was incubated with antimouse horseradish peroxidase-conjugated Ab (Amersham, Arlington Heights, IL). After washing with PBS-T, the blots were developed by chemiluminescence (ECL protein detection reagent; Amer sham).

**Statistical Analysis.** Statistical significance was determined by the Student’s t test.

**RESULTS**

The correlation between the growth inhibition and the dose of TRAIL is illustrated in Fig. 1. After 24 h, Bxpc3 and SW979 cells were most affected, whereas the response of Miapaca2, Panc89, and HPAF was moderate. Panc1 showed a minimal response, and Aspc1 showed no response at all. The IC₅₀ values for Bxpc3, SW979, Panc89, Miapaca2, HPAF, Panc1, and Aspc1 were 12, 20, 33, 48, 66, 95, and >1600 ng/ml TRAIL, respectively.

The time course of the cell death in the cell lines after treatment with TRAIL is shown in Fig. 2. TRAIL-induced cell death was very rapid and was seen already after 2 h. In Bxpc3, Panc89, Miapaca2, and SW979 cells, most of the cells were dead, and only a few cells were still attached to the bottom of the tissue culture dish.
In HPAF and Panc1 cells, the survival rate was 15% and 40%, respectively, compared with their respective untreated cell controls.

Treatment of cells with TRAIL for eight times almost depleted the Bxpc3, Panc89, Miapaca2, and SW979 cells (Fig. 3; Table 2). HPAF showed a significant cell number reduction during the first four treatments, followed by a slight and sustained recovery. In Panc1 cells, the cell number increased

**Table 2** Cell growth after treatment with TRAIL

<table>
<thead>
<tr>
<th>Day</th>
<th>Bxpc3 TRAIL</th>
<th>Control</th>
<th>Panc89 TRAIL</th>
<th>Control</th>
<th>Miapaca2 TRAIL</th>
<th>Control</th>
<th>SW979 TRAIL</th>
<th>Control</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>400</td>
<td>400</td>
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<td>400</td>
<td>400</td>
</tr>
<tr>
<td>3</td>
<td>65 ± 7</td>
<td>555 ± 35</td>
<td>132 ± 9</td>
<td>925 ± 30</td>
<td>53 ± 3</td>
<td>900 ± 40</td>
<td>50 ± 3</td>
<td>700 ± 30</td>
</tr>
<tr>
<td>6</td>
<td>41 ± 7</td>
<td>1,100 ± 100</td>
<td>41 ± 10</td>
<td>3,499 ± 44</td>
<td>83 ± 2</td>
<td>4,112 ± 150</td>
<td>55 ± 7</td>
<td>1,900 ± 21</td>
</tr>
<tr>
<td>9</td>
<td>33 ± 4</td>
<td>3,960 ± 1,500</td>
<td>50 ± 11</td>
<td>10,513 ± 1,650</td>
<td>50 ± 2</td>
<td>6,471 ± 1,000</td>
<td>50 ± 6</td>
<td>5,120 ± 1,000</td>
</tr>
<tr>
<td>12</td>
<td>38 ± 4</td>
<td>11,000 ± 2,300</td>
<td>45 ± 9</td>
<td>12,000 ± 1,000</td>
<td>49 ± 3</td>
<td>10,000 ± 2,100</td>
<td>49 ± 7</td>
<td>11,100 ± 2,000</td>
</tr>
<tr>
<td>15</td>
<td>45 ± 7</td>
<td>Confluent</td>
<td>37 ± 10</td>
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<td>46 ± 4</td>
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<td>48 ± 5</td>
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<tr>
<td>21</td>
<td>50 ± 5</td>
<td>43 ± 4</td>
<td>49 ± 6</td>
<td>45 ± 4</td>
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<tr>
<td>24</td>
<td>45 ± 4</td>
<td>43 ± 7</td>
<td>45 ± 4</td>
<td>46 ± 3</td>
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<td>46 ± 3</td>
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**A. Bxpc3, Panc89, Miapaca2**, and **SW979 cells**

<table>
<thead>
<tr>
<th>Day</th>
<th>HPAF TRAIL</th>
<th>Control</th>
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<th>Control</th>
<th>Apsc1 TRAIL</th>
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<tr>
<td>3</td>
<td>150 ± 21</td>
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<td>2,250 ± 110</td>
<td>4,800 ± 110</td>
<td>1,715 ± 160</td>
<td>2,476 ± 400</td>
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<tr>
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<td>100 ± 13</td>
<td>9,150 ± 210</td>
<td>3,900 ± 1,100</td>
<td>20,300 ± 1,000</td>
<td>2,610 ± 700</td>
<td>5,105 ± 1,000</td>
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<tr>
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<td>350 ± 41</td>
<td>13,600 ± 1,600</td>
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<td>22,150 ± 2,000</td>
<td>7,761 ± 500</td>
<td>9,546 ± 1,600</td>
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<tr>
<td>12</td>
<td>650 ± 72</td>
<td>14,100 ± 710</td>
<td>4,400 ± 700</td>
<td>21,000 ± 2,200</td>
<td>12,079 ± 1,400</td>
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<tr>
<td>15</td>
<td>2,700 ± 500</td>
<td>Confluent</td>
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<td>Confluent</td>
<td>11,986 ± 1,000</td>
<td>13,617 ± 1,500</td>
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<tr>
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<td>3,850 ± 570</td>
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<td>5,600 ± 850</td>
<td>5,600 ± 850</td>
<td>11,986 ± 1,000</td>
<td>13,617 ± 1,500</td>
</tr>
</tbody>
</table>

* P < 0.01.

**B. HPAF, Panc1, and Apsc1 cells**

Fig. 3 The cell growth after treatment eight times with TRAIL. The treatment period was 3 days each time. The cell number was determined every 3 days. X axis represents the number of TRAIL treatments, and Y axis represents the cell number. ○, control; ■, TRAIL treatment; the broken lines represent confluent state of the cells. *, P < 0.01.
slightly but gradually after one treatment and remained stable at around 500,000 cells until the last treatment, when the control cells became confluent (2,100,000 cells). Treatment with TRAIL did not alter the cytological appearance of the cells in any cell line. Aspc1 proved to be refractory also to the repeated TRAIL treatment. The effect of TRAIL on apoptosis, as measured by TUNEL method, is illustrated in Fig. 4. The Bxpc3 cell line, which had 71.5% apoptotic cells, was the most affected, whereas Aspc1 cells with an apoptotic rate of 5.5% were the least responsive. The response correlated closely with the cell death rate of the cell line (Fig. 2). Agarose gel electrophoresis showed that TRAIL induced DNA fragmentation (Fig. 5) in all cell lines. However, in Aspc1 cells, the fragmentation was very weak.

The apoptotic potential of the combined treatment (TRAIL plus actinomycin D) was investigated on the TRAIL-resistant Aspc1 cells. After treatment for 24 h, almost all cells were detached and floated in the flask, whereas only a few cells were found detached after treatment with actinomycin D alone. The survival rate of Aspc1 cells treated with the combined treatment was 47% (actinomycin D, 0.1 μg/ml) or 7.2% (actinomycin D, 1 μg/ml) compared with the cells treated with actinomycin D (either concentration) alone (Table 3). Similar results were found for HPAF (survival rates of 17.9% and 6.7%, respectively), which was moderately sensitive to TRAIL, whereas in Bxpc3, due to the already strong effect of TRAIL alone, the survival rate (6%) could not be further decreased by combination with actinomycin D. The apoptosis rate was significantly higher after combined treatment than it was in cells treated with actinomycin D or TRAIL alone. Western blot analysis demonstrated the presence of FLIP in Aspc1 cells (resistant to TRAIL) treated with TRAIL but not in the cells treated with 0.1 μg/ml actinomycin D (Fig. 6). However, actinomycin D showed no such effect on FLIP in HPAF (moderately sensitive to TRAIL) and Bxpc3 cells (highly sensitive to TRAIL; Fig. 6).

Immunocytochemical examination showed no correlation between TRAIL sensitivity and the expression of DU-PAN 2, CA19-9, B72.3, transforming growth factor α, epidermal growth factor receptor, p16, p53, bcl-2, and bax expression, on the one hand, and the differentiation state of cell lines on the other hand (Table 4). The immunocytochemical results of p53 staining correlate with the reported data (21, 22). There were also no differences in the immunoreactivity between the cells before and after TRAIL treatment.

The expression of all four TRAIL receptors (DR4, DR5,
DcR1, and DcR2) was detected by RT-PCR analysis. The glioma cell line H79, which was used as a positive control, expressed only receptors DR5 and DcR1 (Fig. 7). The TRAIL receptors were also detected by Western blot in all four cell lines (Fig. 8).

DISCUSSION

Previous studies have shown the presence of high affinity receptors to recombinant human TNF-α and IFN-γ in pancreatic cancer cells, which showed no, little, or profound sensitivity to these cytokines (15). Similar growth-inhibitory effects were obtained with the natural human TNF-α and IFN-α (23). TRAIL (also called APO2 ligand), a new and novel member of the TNF superfamily, has induced apoptosis in many transformed cell lines by binding to its death-signaling receptors, DR4 and DR5, and initiating a cascade of events leading to apoptosis in a caspase-dependent fashion (24, 25). Although its death domain-containing receptors are expressed in both normal and cancer cells (3, 26), TRAIL is not cytotoxic to normal cells, although some toxicity to normal human hepatocytes was reported recently (12). This protection has been thought to be due to the presence of the antagonist DcR1 (or TRID) and DcR2 that inhibit TRAIL signaling in normal cells but are absent in most tumor cells (27–29). The DcR1 receptor is a distinct gene product with an extracellular TRAIL-binding and a transmembrane domain but no intracellular signaling domain. Ectopic expression of DcR1 has been shown to protect mammalian cells from TRAIL-induced apoptosis (28).

In some aspects, the effects of TRAIL differ from other TNF family members. Besides being nontoxic to normal cells, it also induces apoptosis in FasL-resistant tumor cells (14) and does not have a graft- versus -host effect but has a favorable graft-versus-tumor effect (4, 6). The resistance of human pancreatic cancer cells to Fas-mediated apoptosis, despite the expression of Fas and FasL in these cells (14), clearly distinguishes the effect of TRAIL from FasL. TRAIL activity in hematological malignancies was not reduced by the overexpression of the multidrug-resistant protein and was not enhanced by 9-cis retinoids, which down-regulate the bcl-2 protein (6). Therefore, TRAIL has been considered a therapeutic drug and has shown growth-inhibitory effects on a variety of mesenchymal and epithelial cancer cell lines (6–11). Although bcl-2, bax caspases, and the overexpression of caspase-1, which causes apoptosis in rat fibroblasts (30), have been identified in pancreatic cancer cells (31, 32), little information is available on the effect of TRAIL on these cells. Based on the unavailability of any therapeutic modalities for pancreatic cancer, its high mortality, and lack of early diagnostic possibilities, TRAIL could provide a unique drug to control this dismal disease.

Realizing the cell heterogeneity of solid tumors including

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**Table 4** Immunocytochemical findings in human pancreatic cancer cells treated with TRAIL.

<table>
<thead>
<tr>
<th></th>
<th>Highly sensitive</th>
<th>Moderately sensitive</th>
<th>Slightly sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bxpc3 Panc89 Miapaca2 SW979</td>
<td>HPAF Panc1 Aspc1</td>
<td>H 2 O</td>
</tr>
<tr>
<td>% apoptosis</td>
<td>71.5 15.8 24.9 13.9</td>
<td>38.0 16.1 5.3</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>+ + + -</td>
<td>+ + + -</td>
<td></td>
</tr>
</tbody>
</table>

* Mod., moderately differentiated; Undif., undifferentiated; Well, well-differentiated.
* The immunoreactivity correlated with the published data (21, 22).
pancreatic cancer, which may determine the cell response to therapeutic agents, we tested the effect of TRAIL on heterogeneous human pancreatic cancer cell lines of different origin and differentiation to examine whether the effect of TRAIL is dependent or independent from the cancer cell type. We used the leucin zipper form of human TRAIL because it causes cell death in a TRAIL-sensitive human adenocarcinoma cell line (8). Our initial scope was to assess the short-term and long-term effect of TRAIL on cell survival.

TRAIL was effective in reducing the cell number and causing apoptosis in all but one cell line. However, these effects varied among the cell lines with a significant difference in the IC$_{50}$ values of TRAIL and its apoptotic property. Bxpc3, Panc89, Miapaca2, and SW979 were very sensitive to TRAIL, whereas the response of HPAF and Panc1 was moderate. Remarkably, Bxpc3 cells, which are resistant to FasL (14), were the most responsive to TRAIL. In the responsive cells, the effect of TRAIL was temporary because the cell number increased after the removal of TRAIL except in Bxpc3 cells, which showed a sustained profound growth inhibition even after the removal of TRAIL. However, repeated treatment caused an almost complete cell loss in Panc1, Miapaca2, Panc89, and SW979 cells and long-lasting growth suppression in Panc1 and HPAF cells. Reasons for the recovery of HPAF and Panc1 cells at a limited rate during the entire treatment period are hard to understand. One can speculate that some cells in these two lines become partially resistant to TRAIL and grow slowly in the presence of TRAIL. It would be interesting to find out whether this partial resistance can be overcome by using additional cytokines, such as TNF-α, which is shown to affect Miapaca2 (33).

Confirming an earlier finding (18), no correlation was found between the effect of TRAIL and the expression of bcl-2, bax, and p53.

Differences in the response of the cell lines to the cytotoxic effect of TRAIL could be due to different factors: (a) an incomplete or mutated receptor (34); (b) ras transformation that makes cells resistant to cell death (35); or (c) the persistence or acquisition of the DcRs DcR1 or DcR2 in malignant cells. However, the presence of the mRNA and protein for the TRAIL receptors supports the lack of correlation between the presence of the TRAIL receptor and its cytotoxic effect (36) and indicates that the apoptotic effect of TRAIL is also governed by other mechanisms. This was highlighted by the response of the TRAIL-resistant Aspc1 cells to a combination of TRAIL and actinomycin D. This observation correlates with findings in melanoma cells, which, like Aspc1 cells, showed a lack of correlation between the presence of the DcRs and their sensitivity to TRAIL (37). Similarly, a high expression of FLIP and a resistance to TRAIL was also found in this melanoma cells (37, 38). Moreover, as in Aspc1 cells, actinomycin D decreased the levels of the FLIP protein within a few hours and increased the susceptibility of the melanoma cells to TRAIL-induced death, although actinomycin D itself did not have any apoptotic or toxic effect on these cells (37). Although these findings suggest an important role of FLIP in the apoptotic pathway of TRAIL, its presence also in the TRAIL sensitive Bxpc3 and HPAF cells and its persistence after actinomycin D treatment complicate the issue. Mechanistic studies are needed to understand this rather complex process. Nevertheless, the results promise an effective therapeutic modality for the treatment of pancreatic cancer resistant to TRAIL.

REFERENCES


Combination of Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) and Actinomycin D Induces Apoptosis Even in TRAIL-resistant Human Pancreatic Cancer Cells


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