ABSTRACT

Fas (APO-1/CD95) is a transmembrane protein of the tumor necrosis factor (TNF) nerve growth factor receptor superfamily that induces apoptosis in susceptible normal and neoplastic cells upon cross-linking by its ligand (Fasl). TNF-related apoptosis-inducing ligand (TRAIL) is a more recently identified member of the TNF superfamily that has been shown to selectively kill neoplastic cells by engaging two cell-surface receptors, DR4 and DR5. Two additional TRAIL receptors (DcR1 and DcR2) do not transmit an apoptotic signal and have been proposed to confer protection from TRAIL-induced apoptosis. We addressed the expression of Fas, DR4, and DR5 in thyroid carcinoma cell lines and in 31 thyroid carcinoma specimens by Western blot analysis and immunohistochemistry, respectively, and tested the sensitivity of thyroid carcinoma cell lines to Fas- and TRAIL-induced apoptosis. Fas was found to be expressed in most thyroid carcinoma cell lines and tissue specimens. Although cross-linking of Fas did not induce apoptosis in thyroid carcinoma cell lines, Fas-mediated apoptosis did occur in the presence of the protein synthesis inhibitor cycloheximide, suggesting the presence of a short-lived inhibitor of the Fas pathway in these cells. Cross-linking of Fas failed to induce recruitment and activation of caspase-8, whereas transfection of a constitutively active caspase-8 construct effectively killed the SW579 papillary carcinoma cell line, arguing that the action of the putative inhibitor occurs upstream of caspase-8. By contrast, recombinant TRAIL induced apoptosis in 10 of 12 thyroid carcinoma cell lines tested, by activating caspase-10 at the receptor level and triggering a caspase-mediated apoptotic cascade. Resistance to TRAIL did not correlate with DcR1 or DcR2 protein expression and was overcome by protein synthesis inhibition in 50% of the resistant cell lines. One medullary carcinoma cell line was resistant to Fas- and TRAIL-induced apoptosis, even in the presence of cycloheximide, and to transfection of constitutively active caspase-8, suggesting a different regulation of the apoptotic pathway. Our observations indicate that TRAIL effectively kills carcinomas that originate from the follicular epithelium of the thyroid gland, by inducing caspase-mediated apoptosis, and may provide a potentially potent therapeutic reagent against thyroid cancer.

INTRODUCTION

Members of the TNF receptor superfamily of cell surface proteins regulate diverse biological processes, including cell activation, proliferation, cytokine production, and programmed cell death (apoptosis). Because transduction of signals that induce apoptosis is the salient functional property of TNF-R1 and Fas (Apo-1/CD95; Ref. 1), the Fas death domain, termed the “death domain,” that is essential for apoptosis signaling, Upon engagement by their respective ligands (TNF-α and FasL), TNF-receptor 1 and Fas recruit adaptor molecules (TRADD and FADD, respectively) through their death domains and activate a cascade of cysteine proteases (caspases), the proteolytic activity of which induces apoptosis (2). Fas-mediated apoptosis plays an important role in the homeostasis of the immune system (3, 4) and provides one mechanism for T cell-mediated cytotoxicity (5). Recombinant FasL, and cross-linking anti-Fas antibodies have been used to kill tumor cells in vivo, but the results have often been disappointing because of the inherent resistance to Fas-mediated cytotoxicity of many tumor cell types (6, 7).

Recently, another member of the TNF family, the TRAIL (8) or Apo-2 L (9), has been identified. TRAIL interacts with two newly discovered DRs, DR4 (or TRAIL-R1; Ref. 10) and DR5 (or TRAIL-R2; Refs. 11–14). Transfection experiments have shown that both DR4 and DR5 can initiate caspase-mediated apoptosis (10–12). Unlike FasL, the expression of which is normally limited to cells of the immune system (15) and a few immune-privileged sites (16–18), TRAIL expression has been detected in a wide range of normal fetal and adult tissues (9). These findings suggest the existence of a protective mechanism against TRAIL-mediated cytotoxicity in normal cells, which is supported by the observations that TRAIL can induce apoptosis in transformed and malignant cells (10), but not in normal cells (12). Because of this selectivity, TRAIL represents a potentially promising new candidate for cancer therapy.

Thyroid cancer is diagnosed in over 11,000 new patients each year in the United States. Although radioactive iodine remains an efficient treatment for the subset of differentiated tumors that have retained the ability to accumulate it, a poor prognosis is still associated with less differentiated, anaplastic, and medullary carcinomas. Fas is expressed in normal thyrocytes (19–23), where its expression is strongly up-regulated during the course of Hashimoto’s thyroiditis, possibly under the influence of lymphocyte-derived cytokines, leading to apoptotic suicide/fratricide and contributing to the destruction of the gland in this disease (19, 24). TRAIL receptors DR4 and DR5 are also expressed in normal thyrocytes (25). However, the functional status of the Fas and TRAIL apoptotic pathways in thyroid carcinoma cells is still poorly studied.

We therefore addressed the feasibility of activating Fas- and TRAIL-associated death pathways as a means to eliminate thyroid carcinoma cells. We found that Fas, DR4, and DR5 are expressed in most cases of thyroid carcinoma in vitro and in vivo. Thyroid carcinoma cell lines were resistant to Fas cross-linking, yet sensitive to TRAIL, which triggered a caspase cascade originating at caspase-10. We conclude that TRAIL may provide a potential new therapeutic modality for thyroid cancer.

MATERIALS AND METHODS

Human Tissues

Archival formalin-fixed and paraffin-embedded thyroid specimens from 31 patients (9 males and 22 females) with thyroid carcinomas, ages 33–76 years (mean ± SD, 50 ± 11), were retrieved retrospectively from the files of the Pathology Department, University of Athens, Greece. They represented 18 papillary, 5 follicular, 2 oxyphilic (Huerthle), 1 anaplastic, and 5 medullary carcinomas.

As controls, specimens of normal thyroid tissue were obtained from the contralateral lobe of eight thyroid glands removed surgically for a nodule from spontaneously euthyroid patients. All thyroid specimens were removed from...
patients followed at the Endocrine Unit of the Evgenidion Hospital, Athens, Greece. All studies on patient material were conducted in accordance with the Declaration of Helsinki principles and Institutional Review Board policies.

**Cell Lines**

Twelve previously described thyroid carcinoma cell lines were used in this study. The papillary thyroid carcinoma cell lines BHP-2, BHP-5, BHP-7, BHP-10, BHP-14, and BHP-17 were generous gifts of Dr. Jerome M. Hershman (West Los Angeles Veterans Affairs Medical Center, Los Angeles, CA). The NPA, FRO, WRO and ARO cell lines (generous gifts of Dr. James A. Fagin, University of Cincinnati School of Medicine, Cincinnati, OH) have been described previously (26). The SW579 cell line, derived from a poorly differentiated human thyroid adenocarcinoma (poorly differentiated carcinoma with nuclear features of papillary carcinoma and squamous differentiation), the medullary carcinoma TT and the T-cell lymphoma H9 cell lines were purchased from American Type Culture Collection (Manassas, VA). Additionally, a primary culture of normal human thyrocytes, isolated from healthy thyroid tissue obtained during thyroectomy, was a generous gift of Dr. G. P. Chrousovs (NIH, Bethesda, MD). All cells were grown in DMEM (BioWhittaker, Walkersville, MD) with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (Life Technologies, Inc., Gaithersburg, MD), unless stated otherwise.

**Materials**

The Vectastain Elite ABC kit for immunohistochemistry was obtained from Vector laboratories, Inc. (Burlingame, CA); human recombinant His-tagged soluble TRAIL from Bionol (Plymouth Meeting, PA); Goat polyclonal antibodies for DR4, DR5, DcR1, actin, and rabbit polyclonal antibody for caspase-3, as well as the corresponding blocking peptides, from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal antibody for caspase 8 and FADD and rabbit polyclonal antibody for DR4, DR5, DcR1, actin, and rabbit polyclonal antibody for caspase-3, as well as the corresponding blocking peptides, from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal antibody for caspase 8 and FADD and rabbit polyclonal antibody for caspase-10 from Upstate Biotechnology (Lake Placid, NY); the anti-DR2 rabbit polyclonal antibody from Imgenex (San Diego, CA); rabbit anti-Fas Ab-1 from Oncogene Research (Cambridge, MA); blocking peptide used in immunohistochemistry and corresponding to amino acid residues 321–335 of Fas (25 μg/ml) from Oncogene Research (Cambridge, MA); anti-Fas CH11 antibody from Panvera (Madison, WI); 3-diaminobenzidine, cycloheximide, MTT, IFN-γ, and TNF-α from Sigma Chemical Co. (St. Louis, MO); the in situ cell death detection kit-Fluorescence, Complete-TM mixture of proteinase inhibitors, IgG-free normal horse serum, and SDS from Life Technologies, Inc. (Gaithersburg, MD); and the Enhanced Chemiluminescence (ECL) kit, which includes the peroxidase-labeled antimonute and antirabbit secondary antibodies, from Amersham (Arlington Heights, IL).

**Immunohistochemistry**

Immunohistochemical detection of Fas, DR4, and DR5 was performed as described previously (19). The primary antibodies, anti-Fas (1:40 dilution), anti-DR4 (1:100 dilution), and anti-DR5 (1:50 dilution), respectively, were used in the presence or absence of a 10-fold excess of the corresponding blocking peptides. Positive staining was evaluated subjectively by two independent observers.

**Survival and Death Assays**

**MTT Colorimetric Assay.** Cells were plated in 24-well plates and grown to 70–80% confluence. Subsequently, the cells were washed in HBSS and incubated for 18 h with the Fas-activating antibody CH-11 (500 ng/ml) or r-TRAIL (0.5, 1, or 2 μg/ml), in serum-free DMEM medium at 37°C. In some experiments, cycloheximide (10 μg/ml) was added to inhibit protein synthesis. In other experiments, the cells were pretreated with IFN-γ (500 units/ml) or TNF-α (50 ng/ml) or both, for 48 h in serum-free medium. At the end of the 18-h treatment with anti-Fas or r-TRAIL, the cells were incubated with 1 mg/ml MTSS (Sigma) in fresh media for 4 h at 37°C. Then, a mixture of isopropanol and 1 N HCl (24:1, v/v) was added under vigorous pipetting to dissolve the formazan crystals. Dye absorbance (A) in viable cells was measured at 570 nm, with 630 nm as a reference wavelength. Cell death was estimated with the formula:

\[
\% \text{ specific death} = \left( \frac{A \text{ (untreated cells)} - A \text{ (treated cells)}}{A \text{ (untreated cells)}} \right) \times 100
\]

Each experiment was repeated at least three times. Every experimental condition was repeated at least in sextuplicate wells for every experiment.

**TUNEL Method.** SW579 and FRO cells were treated or not with 1 μg/ml r-TRAIL, for 16 h. Air-dried cytopsins of cells were labeled with the in situ cell death kit-Fluorescence (Boehringer Mannheim), following the instructions of the manufacturer, and were viewed with a Zeiss standard fluorescence microscope equipped with an epifluorescence illuminator and FITC narrow-band filter.

**Western Blotting Analysis.** Immunoblotting analysis was performed as described previously (6). The proteins were visualized with the enhanced chemiluminescence technique (Amersham Pharmacia Biotech, Piscataway, NJ).

**Activation and Precipitation of the Fas Signaling Complex**

Immunoprecipitation of Fas and the associated caspase 8 was carried out in SW579 and the H9 cell lines. Briefly, 10^5 cells, either unstimulated or treated with 1 μg/ml biotinylated anti-APO-1 (Kamiya, Seattle, WA) for 20 min, were harvested and lysed as before. All lysates were subsequently incubated at 4°C with 1 μg/ml of biotinylated mouse anti-Fas overnight and with streptavidin-agarose (Upstate Biotechnology) for 2 h. The immunoprecipitates were electrophoresed in SDS-PAGE and assayed for the presence of caspase 8, as described previously.

**Transfection of Thyroid Carcinoma Cells with the CD8-Caspase 8 Molecular Chimera**

SW579 and TT cells were plated in 12-well plates, grown to 70–80% confluence, and transfected with the pCEFL eukaryotic expression vector carrying the sequence of human CD8 fused to wild-type caspase 8 or to an inactive caspase 8 mutant (27) or with the empty vector (generous gifts of Dr. M. Lenardo, NIH, Bethesda, MD). Twenty-four h later, cell death was assessed with MTT.

**Caspase Cleavage**

To study the potential involvement of caspases in TRAIL-mediated apoptosis, SW579 cells were treated with 1 μg/ml r-TRAIL for 0, 1 and 4 h. Caspase cleavage and, hence, activation, was detected by Western blotting, that was performed as described previously.

**TRASC Assay.** SW579 cells were treated with 1 μg/ml r-TRAIL for 30 min or left untreated. Subsequently, they were harvested and lysed in TRASC buffer [20 mM HEPES (pH 7.4), 200 mM NaCl, and 1% [g]al] supplemented with proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin). The samples were cleared by microcentrifugation (14,000 rpm for 30 min at 4°C) and incubated with 1 μg/ml r-TRAIL for 4 h at 4°C. The His-tagged r-TRAIL and all associated proteins were precipitated with Ni^{2+}-conjugated agarose beads (Qiagen, Valencia, CA) for 2 h at 4°C, electrophoresed in a 12% SDS-PAGE, electroblotted onto nitrocellulose membranes, and detected by immunoblotting with the corresponding antibodies.

**Statistical Analysis**

Quantitative comparisons were examined with the ANOVA method, followed by Duncan’s test. Statistical significance was set at 0.05.

**RESULTS**

**Expression of Fas in Thyroid Carcinomas and Susceptibility of Thyroid Carcinoma Cell Lines to Fas-mediated Apoptosis.** Fas expression was detected in normal thyroid tissue, as shown previously (19). In neoplastic tissue, Fas was present in 18 of 18 papillary (Fig. 1a), 4 of 5 follicular, 2 of 2 Hurthle, 1 of 1 anaplastic, and 4 of 5 medullary (Fig. 1b) carcinomas. In our study, Fas expression was not associated with prognostic parameters, such as patient age, tumor size, and extension and recurrence of disease. All immunostaining was inhibited by preincubation of the antibody with the corresponding blocking peptide, and immunoblotting analysis revealed Fas expression in cultured normal thyrocytes and in all thyroid carcinoma cell lines tested (Fig. 1c).

We have shown previously that the papillary thyroid carcinoma cell line SW579, which is resistant to the apoptosis-inducing anti-Fas antibody CH11, can be rendered sensitive by cycloheximide (28). Because our present observations indicate that Fas is expressed in thyroid carcinomas of all histological subtypes, we tested the susceptibility to Fas-mediated death signals of the follicular carcinoma cell line WRO, the
anaplastic carcinoma cell lines FRO and ARO, and the medullary carcinoma cell line TT. All four cell lines were resistant to cell death induced by the CH11 antibody. However, cycloheximide sensitized the follicular and anaplastic carcinoma cell lines (P < 0.005 in all cases), but not the medullary carcinoma (P = 0.37), to Fas-mediated apoptosis (Fig. 2). Taken together, these data suggest that in thyroid carcinomas originating from the follicular epithelium, the Fas apoptosis pathway is blocked by a short-lived inhibitory protein, consistent with previous observations on normal thyroid follicular cells (22, 23).

Caspase 8 Is Not Recruited to Fas in Thyroid Carcinomas. The recruitment of caspase 8 to Fas and its subsequent proteolytic autoactivation are necessary steps in the Fas-mediated cell death. To determine whether failure to transduce signals that initiate apoptosis after engagement of Fas in thyroid carcinomas is attributable to a defect of caspase 8 recruitment, we treated SW579 thyroid carcinoma cells with an anti-Fas monoclonal antibody and immunoprecipitated Fas and its associated proteins. Human T lymphocytic H9 cells, where caspase 8 recruitment to cross-linked Fas has been shown (29), served as a positive control. We found that upon activation of Fas, the two isoforms of the full-length caspase 8 (caspase-8/a and caspase 8/b), as well as the cleavage intermediate p43, which is generated by removing the p10 active subunit from the zymogen, can be found in association with the receptor in H9 cells but not in SW579 cells (Fig. 3a). These data suggest that the absence of caspase 8 activation at the level of the receptor could account for the resistance of thyroid carcinomas to Fas cross-linking.

An Active CD8-Caspase 8 Fusion Protein Induces Apoptosis in Fas-resistant Thyroid Carcinoma Cells. We next addressed whether signaling events downstream of caspase-8 activation might be blocked in thyroid carcinoma cells. Previous studies have shown that expression of a cDNA encoding the full-length caspase-8 does not induce apoptosis efficiently (27), suggesting that the proenzyme does not spontaneously undergo proteolytic autoactivation. However, a chimeric construct composed of caspase-8 cDNA sequences fused to a cDNA encoding the cell surface protein CD8, which forms a disulfide-linked homodimer, has been shown to be a potent inducer of apoptosis in Jurkat T cells, probably because of autoactivation of caspase-8 by oligomerization at the cell membrane (27). We therefore compared the effect of expressing the CD8-caspase 8 chimeric construct (CD8-C8), its active site mutant (CD8-C8mut), and the empty vector on the survival of SW579 and TT thyroid carcinoma cells. Expression of the enzymatically active construct induced cell death (P < 0.005 versus both the empty vector and the mutant construct) in papillary SW579 cells (Fig. 3b) but not in medullary TT cells (Fig. 3c). These observations are consistent with the presence of at least one inhibitor upstream or at the level of caspase 8 activation in papillary thyroid carcinoma cells, whereas in medullary carcinoma cells, the resistance to apoptosis lies downstream of caspase 8.

Cytokines Sensitize Thyroid Carcinoma Cells to Fas-mediated Cell Death. Cytokines, including IFN-γ and TNF-α, are produced by immune cells that infiltrate thyroid carcinomas and hence may play a role in regulating the survival of these tumors. We therefore investigated whether cytokines could sensitize thyroid carcinoma cells to Fas-mediated apoptosis. We treated SW579 thyroid carcinoma cells with IFN-γ and TNF-α, both of which are known to upregulate Fas expression (17, 30), and found that these cytokines induced apoptosis in a dose-dependent manner (Fig. 4a). To determine whether this effect was mediated by Fas, we measured apoptosis in cells treated with the anti-Fas antibody CH11. As shown in Fig. 4b, the addition of CH11 antibody to IFN-γ and TNF-α-treated cells completely blocked apoptosis, indicating that these cytokines sensitize thyroid carcinoma cells to Fas-mediated apoptosis.

Fig. 1. a and b, immunohistochemical detection of Fas in a papillary (a, ×240) and a medullary (b, ×225) thyroid carcinoma. c and d, Western blot analysis for Fas in lysates derived from normal thyrocytes and a panel of thyroid carcinoma cell lines (c). Actin protein levels are shown for normalization (d).

Fig. 2. Survival of WRO (a), FRO (b), ARO (c) and TT (d) thyroid carcinoma cells treated with CH11 (500 ng/ml), cycloheximide (10 μg/ml), or both for 18 h. Values represent the mean of sextuplicate measurements; bars, SD. *, statistical significance (P < 0.05).
in the effort of the immune system to control malignancy. To determine whether cytokines that compose part of the arsenal of the immune system against tumors might alter thyroid carcinoma sensitivity to Fas, SW579 and WRO cells were triggered with anti-Fas mAb after treatment with IFN-γ, TNF-α, or a combination of both for 48 h. Both cell lines were sensitized to Fas-mediated cell death by cytokine pretreatment. In the papillary SW579 cell line, IFN-γ had a strong sensitizing effect when applied either alone or in combination with TNF-α (Fig. 4a). One possible explanation for this effect was the strong up-regulation of Fas protein levels by IFN-γ (Fig. 4b). Conversely, in the follicular WRO cell line, the sensitizing effect was more prominent with TNF-α and was enhanced when TNF-α and IFN-γ were combined (Fig. 4c).

Expression of Fas in thyroid carcinoma cell lines. FTU cells were exposed to cytokine treatment for 48 h, followed by treatment with the anti-Fas antibody. Survival of SW579 and WRO cells pretreated with IFN-γ, TNF-α, or both for 48 h. Values represent the means of sextuplicate measurements; bars, SD.

Values represent the means of sextuplicate measurements; bars, SD.

Expression of Decoy TRAIL Receptors Is an Unlikely Explanation for Thyroid Carcinoma Cell Resistance to TRAIL-mediated Cell Death. To address the possible mechanism underlying the resistance to TRAIL-induced apoptosis that was observed in normal thyrocytes and the WRO and TT cell lines, expression of the decoy TRAIL receptors DcR1 and DcR2 was assessed by Western blot analysis. Decoy receptor expression did not correlate with cell resistance to TRAIL. DcR1 was expressed in all carcinoma cell lines but not in normal thyrocytes and the WRO and TT cell lines. Only rare untreated cells displayed TUNEL staining (Fig. 7).

Fig. 4. Effect of cytokines on thyroid carcinoma sensitivity to Fas. a, survival of SW579 cells that were pretreated with IFN-γ, TNF-α or both for 48 h and, subsequently, treated with the anti-Fas antibody CH11 for 18 h. Values represent the means of sextuplicate measurements; bars, SD. b, an increase in Fas expression at the protein level was found in SW579 cells after treatment with IFN-γ, alone or in combination with TNF-α, for 48 h. TNF-α alone had only a modest stimulating effect. Actin protein levels are shown for normalization. c, survival of WRO cells that were pretreated with IFN-γ, TNF-α, or both for 48 h and, subsequently, treated with the anti-Fas antibody CH11 for 18 h. Values represent the means of sextuplicate measurements; bars, SD.

Expression of TRAIL Receptors DR4 and DR5 in Thyroid Carcinomas and Susceptibility to TRAIL-mediated Cell Death in Thyroid Carcinoma Cell Lines. We next addressed the expression of two other apoptosis-inducing receptors, DR4 (TRAIL-R1) and DR5 (TRAIL-R2), in thyroid tissues and cultured cells. Strong DR4 immuno-reactivity was observed in normal thyroid tissue (Fig. 5a) and in 18 of 18 papillary (Fig. 5b), 5 of 5 follicular, 2 of 2 oxyphilic, 1 of 1 anaplastic (Fig. 5c), and 4 of 5 medullary carcinoma specimens. DR5 immunoreactivity was found in normal thyroid tissue (Fig. 5d) and in 11 of 18 papillary (Fig. 5e), 3 of 5 follicular (Fig. 5f), 2 of 2 oxyphilic, 1 of 1 anaplastic, and 2 of 5 medullary carcinoma specimens. Immunostaining of both receptors was consistent with cytoplasmic and cell surface expression. DR4 displayed stronger and more widespread immunoreactivity than DR5, both within and among tumor specimens, as well as in normal tissue. Preincubation of each antibody with the respective blocking peptide completely abolished the immunostaining, confirming the specificity of the staining.

Western blot analysis revealed DR4 expression in normal thyrocytes in vitro and in all thyroid carcinoma cell lines tested (Fig. 5g). DR5 expression was detectable but weak in cultured normal thyrocytes and was stronger in the carcinoma cell lines (Fig. 5h).

r-TRAIL was observed to induce cell death in 10 of 12 thyroid carcinoma cell lines (Fig. 6). All 8 papillary and the 2 anaplastic cell lines tested were sensitive to TRAIL-induced cell death, whereas the follicular and the medullary cell lines were resistant. Consistent with a previous study (25), we found that normal thyrocytes were resistant to TRAIL. TRAIL-induced cell death was apoptotic, as shown by the presence of positive TUNEL staining of TRAIL-treated SW579 and FRO cells. Only rare untreated cells displayed TUNEL staining (Fig. 7).
DcR2 expression (Fig. 8b). Therefore, DcR2 is unlikely to contribute to the resistance of normal thyrocytes or TT carcinoma cells to TRAIL.

Interestingly, cycloheximide treatment sensitized WRO (P = 0.005), but not TT cells (P = 0.1), to TRAIL-induced apoptosis (Fig. 9). These data suggest the existence of inhibitory protein-dependent and -independent mechanisms of resistance to TRAIL in thyroid carcinomas.

Identification of a TRASC. The involvement of caspases in TRAIL-induced cell death in thyroid carcinomas was addressed by Western blot analysis of lysates from SW579 cells treated with 1 μg/ml r-TRAIL for 0, 1, and 4 h. Cleaved (activated) caspase-10 was detected in total cell lysates as early as 1 h after r-TRAIL addition to the cell cultures. Caspase-8 and caspase-3 were observed to be activated 4 h after addition of TRAIL (Fig. 10), consistent with the notion that TRAIL triggers a caspase cascade in thyroid carcinoma cells. In an attempt to identify the apical components of this cascade that interact with the TRAIL receptor(s), SW-579 cells were triggered with His-tagged r-TRAIL, and the associated signaling complex was precipitated and subjected to SDS-PAGE. All TRAIL receptors were precipitated by r-TRAIL. In addition, stimulation of SW-579 cells with r-TRAIL induced the recruitment of caspase-10, but not caspase-8, to the receptor-ligand complex (Fig. 11). This suggests that caspase-10 is the apical caspase in the apoptotic cascade triggered by TRAIL in thyroid carcinoma cells. Because we did not detect FADD recruitment to the signaling complex (Fig. 11), it is possible that another adaptor molecule, which displays preference for caspase-10 recruitment, is involved in TRAIL signaling.

DISCUSSION

In the present study, we addressed thyroid carcinoma sensitivity to Fas- and TRAIL receptor-mediated cell death. We found that Fas as well as the TRAIL receptors DR4 and DR5 are broadly expressed in thyroid carcinomas and thyroid carcinoma cell lines. However, the majority of the cell lines tested displayed a different response to stimulation by anti-Fas antibody and TRAIL. Whereas thyroid carcinoma cells were resistant to Fas-mediated apoptosis, ligand-mediated engagement of TRAIL receptors DR4 and DR5 induced apoptotic cell death in thyroid carcinoma cells.
death in 10 of 12 thyroid carcinoma cell lines studied. With the exception of medullary carcinoma cells, resistance to Fas- and TRAIL-induced apoptosis could be overcome by inhibition of protein synthesis.

Because the net rate of carcinoma growth results from the difference between the rates of proliferation and cell death, inhibition of apoptotic death favors cancer progression. Fas-mediated apoptosis is a key mechanism of T cell-mediated cytotoxicity against neoplastic cells (5), and its inhibition has been shown to contribute to tumor progression and metastasis (30). Some tumors have been proposed to resist Fas-mediated cell death by reducing cell surface expression of Fas (31). In the present study, we show that despite expression in most thyroid carcinomas, Fas cannot recruit and activate caspase 8, which is a necessary step for the transduction of the apoptotic signal. Our data suggest that at least two mechanisms may account for resistance to Fas in thyroid carcinoma cells. One may involve the presence of a short-lived inhibitory protein(s), because the protein synthesis inhibitor cycloheximide sensitized papillary, follicular, and anaplastic carcinoma cell lines to Fas-mediated cell death. Such a mechanism has been suggested to confer resistance to Fas in normal human thyrocytes (22). Because expression of a constitutively active caspase-8 construct bypassed the inhibition and induced cell death in papillary carcinoma cells, it is possible that the short-lived inhibitor interferes with the activation of caspase-8 at the Fas-FADD complex. FLIP (32) is an antiapoptotic protein that competes with caspase-8 for binding to FADD and had been shown recently to provide a mechanism for tumor escape from T-cell immunity in vivo (33–35). FLIP is present in thyroid carcinoma cells both in vivo and in vitro and communique-pitizes with Fas in SW579 cells.4 Another inhibitory protein, FAP-1, has been implicated recently in resistance to Fas-mediated apoptosis in normal thyrocytes (36). By contrast, a different antiapoptotic mechanism may protect the medullary carcinoma cell line TT, where neither cycloheximide nor expression of constitutively active caspase-8 could facilitate or induce apoptotic cell death.

Thyroid carcinoma cells can become sensitive to Fas-mediated cell death after treatment with IFN-γ and TNF-α. Although these cytokines may stimulate the apoptotic pathway at various levels, one direct effect of IFN-γ was a strong increase in Fas expression at the protein level. IFN-γ is produced by Th1 lymphocytes, which express FasL and play a major role in regulating cell-mediated immunity. At least one effect of Th1-derived cytokines may be to facilitate tumor cell elimination by cyotoxic T cells. Consistent with this notion, numerous studies have suggested that lymphocytic infiltrates, which are a common finding in papillary carcinomas and are sometimes intense enough to be diagnosed as Hashimoto’s thyroiditis, are associated with a more favorable prognosis (37–42).

Unlike the constitutive functional inhibition of Fas-mediated apoptosis, the death pathway associated with TRAIL receptors could be activated by TRAIL in 10 of 12 thyroid carcinoma cell lines. It is noteworthy that the two anaplastic carcinoma cell lines were sensitive to TRAIL, because anaplastic carcinomas are associated with the worst prognosis among thyroid malignancies. Several of the thyroid carcinoma cell lines used in this study, including FRO and ARO, have been shown to lack p53 expression (43) or harbor p53 mutations (44). Although such functional inactivation of p53 confers resistance to chemotherapeutic drugs and irradiation (45), it did not protect these cells from TRAIL-mediated cell death, suggesting that the death pathway associated with TRAIL functions independently of the p53 status and may thereby offer an advantage over current therapeutic modalities for poorly differentiated thyroid carcinomas.

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4 N. Mitsiades, unpublished observations.
TRAIL-induced cell death was associated with early activation of caspase 10, followed by activation of caspase-8 and caspase-3. A previous study has reported the interaction of caspase-10 with DR4 and DR5, upon transfection of the respective constructs into 293T cells (11). Our study is the first to show that endogenous cellular caspase-10 is recruited to the TRAIL receptor(s) under physiological conditions, upon cross-linking with TRAIL in SW-579 cells. The observation that caspase-10 activation precedes that of caspase-8 is consistent with the possibility that caspase-10 transactivates caspase 8 to amplify the apoptotic signal, as proposed, based on the structure of the caspase-10 active site (46). This finding indicates that endogenous caspase-8 can be activated in thyroid carcinoma cells without requiring inhibition of protein synthesis, underscoring the notion that events upstream of caspase-8 activation are blocked within the Fas pathway.

Identification of anticancer agents that selectively target neoplastic cells and spare normal tissues is one of the ultimate goals of cancer research. Fas is unlikely to provide a useful target for selective elimination of tumors cells for two reasons: (a) many tumor cells are resistant to Fas-mediated cell death, as shown in the present study; and (b) engagement of Fas by a systemically administered activating antibody in mice resulted in severe hepatic toxicity and death (47). Similar to anti-Fas mAb, systemic TNF-α administration has been complicated by prohibitive toxicity (48). By contrast, administration of recombinant TRAIL has been found to be nontoxic for normal cells both in vitro and in vivo (49, 50). Unlike FasL, which is normally expressed only by activated immune cells and a few immune-privileged sites (17, 18), TRAIL is widely expressed in the human body (9) and must therefore be nontoxic to most normal tissues. The selectivity of the cytotoxic activity of TRAIL makes it a potentially powerful candidate for a novel anticancer therapeutic modality and simultaneously poses the interesting question why should malignant cells be sensitive to an agent to which their normal counterparts are resistant?

The discovery of additional TRAIL receptors that cannot transduce an apoptotic signal may be part of the answer to this question. TRAIL-R3 (TRID, DcR1; Refs. 11, 12, 14, and 51) lacks transmembrane and cytoplasmic domains and remains attached to the cell surface via a link to the cell surface glycolipid glycosyl phosphatidylinositol. TRAIL-R4 (TRUNDD, DcR2; Refs. 52–54) contains a cytoplasmic domain with a nonfunctional death domain. Overexpression of either of these proteins protected cells from TRAIL cytotoxicity (11, 12, 53), suggesting that they are naturally occurring dominant-negative forms of TRAIL receptors. Although it is generally assumed that they exert their antiapoptotic action by interfering with the binding of TRAIL to DR4/DR5, it is possible that they have other functions, and in fact, DcR2 has been shown to activate nuclear factor-κB (52). We have found that despite expression of both decoy receptors, thyroid carcinoma cells are sensitive to TRAIL. This result is consistent with recent studies in melanoma and breast carcinoma cell lines (55, 56) and suggests that the regulation of TRAIL-induced apoptosis by the growing family of TRAIL receptors is more complex than originally thought.

Resistance to TRAIL was found in two thyroid carcinoma cell lines in our investigation, and similar to the observed resistance to Fas, two types of resistance to TRAIL were uncovered. WRO cells were sensitized to TRAIL, as they were to Fas, by cycloheximide, suggesting a role for a short-lived apoptosis inhibitor(s). Because most of the Fas-resistant cell lines were sensitive to TRAIL, it is possible that either the inhibitor of the Fas pathway is distinct from that of the TRAIL pathway, or that the former pathway is more sensitive to the same inhibitor than the latter. Interestingly, neither IFN-γ nor TNF-α alone or in combination had any effect on TRAIL resistance in WRO cells (data not shown). The role of FLIP as an inhibitor of apoptosis induced by DR4/DR5 was initially shown by transfection experiments (32), but more recent studies have shown no correlation between endogenous FLIP protein levels and resistance to TRAIL (57). In contrast to WRO cells, cycloheximide had no effect on the resistance of medullary TT cells to TRAIL-mediated apoptosis. Taken together, our data suggest that antiapoptotic protein(s), which are present in carcinomas originating from the thyroid follicular epithelium (papillary, follicular, and anaplastic), confer resistance to apoptosis induced by the death receptors Fas and DR4/DR5, whereas a different mechanism may apply in at least some medullary carcinomas.

Our observations provide evidence that despite its expression in thyroid carcinomas, Fas is incapable of inducing apoptosis in these tumors, attributable, at least in part, to its inability to recruit and activate caspase-8, which may possibly be related to the presence of a short-lived inhibitor(s). By contrast, TRAIL is an efficient inducer of apoptosis in...
carcinoma cell lines originating from the follicular thyroid epithelium, by recruiting and activating caspase-10 and the corresponding downstream caspase cascade. Recombiant TRAIL may therefore provide a promising candidate therapeutic reagent for thyroid cancer.

ACKNOWLEDGMENTS

We thank Dr. Jerome M. Hersham (West Los Angeles Veterans Affairs Medical Center, Los Angeles, CA) and Dr. James A. Fagin (University of Cincinnati School of Medicine, Cincinnati, OH) for providing thyroid carcinoma cell lines. Dr. G. C. Chrousos (National Institutes of Health, Bethesda, MD) for the primary culture of normal thyocytes, and Dr. M. Lernardo (National Institutes of Health, Bethesda, MD) for the caspase-8 plasmids.

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Thyroid Carcinoma Cells Are Resistant to FAS-mediated Apoptosis But Sensitive to Tumor Necrosis Factor-related Apoptosis-inducing Ligand

Nicholas Mitsiades, Vassiliki Poulaki, Sophia Tseleni-Balafouta, et al.

Cancer Res 2000;60:4122-4129.

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