TRAIL Death Pathway Expression and Induction in Thyroid Follicular Cells

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To determine whether programmed cell death in thyroid follicular cells can be related to activation of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) pathway, we examined the expression and function of this pathway in primary thyroid follicular cells and a papillary thyroid carcinoma cell line in vitro. Despite the expression of TRAIL receptors death receptor 4 and death receptor 5, purified TRAIL could not induce programmed cell death (PCD) in any of the thyroid follicular cells examined. However, pre-incubation with cycloheximide before TRAIL facilitated the induction of rapid and massive PCD. This suggested that despite the presence of a labile inhibitor of the TRAIL pathway, TRAIL could mediate PCD under appropriate conditions. To determine whether there were sources of TRAIL in the thyroid that could interact with thyroid follicular cell TRAIL receptors, RNase protection assays were used to determine TRAIL mRNA expression. TRAIL message was expressed in intrathyroidal lymphocytes isolated from a patient with thyroiditis, and unexpectedly, thyroid follicular cells themselves could be induced to express abundant TRAIL message in the presence of the inflammatory cytokines interferon γ, tumor necrosis factor α, and interleukin 1β. Furthermore, the papillary thyroid carcinoma cell line could be induced to kill the TRAIL-sensitive lymphoma cell line BJAB through a TRAIL-dependent mechanism.

PCD may play an important role in thyroid homeostasis and disease. Morphological changes indicative of PCD have been observed in normal thyroid glands and appear at increased frequency in thyroid tissue affected by chronic autoimmune thyroiditis (7–9). PCD is also a mechanism by which cytotoxic T lymphocytes can destroy thyrocytes, which in turn may lead to hypothyroidism (7, 8). In contrast, suppression of PCD may contribute to thyroid proliferative diseases including goiter, cancer, and Graves’ disease (8, 9). However, little is known about the mechanisms and regulation of apoptotic signaling in thyroid cells. Defining the signaling components of PCD present in thyroid follicular cells is important because it may provide insights into potential pathogenic mechanisms and might lead to the development of pharmacological interventions for treatment of thyroid disease. TRAIL (Apoo-2L) is a recently characterized member of the family of PCD-inducing ligands that includes TNF-α and CD95L (FasL) (10–12). TRAIL, like TNF-α and CD95L, has both membrane-bound and soluble forms (10) and acts through type I membrane receptors that signal PCD through a cytosplasmic death domain (12, 13). TRAIL expression by activated T lymphocytes suggests that it may be involved in cell-mediated cytotoxicity (14). Activated T lymphocytes are also the primary cells shown to be susceptible to TRAIL-induced PCD (14–16), suggesting a role in limiting the immune response. In contrast to normal primary cells, numerous tumors of diverse tissue origin are susceptible to TRAIL (17, 18). The known TRAIL death-inducing receptors include DR4 (TRAIL-R1) (19, 20) and DR5 (TRAIL-R2, TRICK2, KILLER/DR5) (20–23). In addition, two TRAIL receptors that do not signal PCD have recently been characterized and have been shown to inhibit DR4- and DR5-mediated death. They are TRID (DeR1, TRAIL-R3, LIT) (21, 22, 24, 25) and TRUNDD (TRAIL-R4, DeR2) (26–28). TRID and TRUNDD both act as decoy receptors and dominant-negative inhibitors of TRAIL-induced cell death (21, 22, 26). TRID is a glycosylphosphatidylinositol-anchored protein incapable of intracellular signaling due to the lack of a cytosplasmic tail (22). TRUNDD has a truncated death domain. TRAIL, DR4, DR5, and TRUNDD each have a broad tissue distribution, suggesting that regulation may also occur at points beyond expression levels of ligand and receptor.

Recently thyrocyte “fraticide” through PCD signaling by the CD95L–CD95 “ligand/receptor pair” has been advanced as an explanation for thyrocyte destruction in thyroiditis (29). Whereas this concept is questionable due to technical reasons (30), PCD does appear to be important in this disease. TRAIL and its two death signaling receptors, DR4 and DR5, are closely related to CD95L and CD95, respectively (10, 21), suggesting similar mechanisms of death signaling. This prompted us to investigate the expression and regulation of TRAIL, DR4, and DR5 in the thyroid. TRAIL is capable of inducing PCD in

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† The abbreviations used are: PCD, programmed cell death; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; IFN-γ, interferon γ; TNF-α, tumor necrosis factor α; IL-1β, interleukin 1β; PARP, poly(ADP-ribose) polymerase; DR, death receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; CHX, cycloheximide; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.
thyroid follicular cells, but only in the presence of the protein synthesis inhibitor cycloheximide. From these results, we conclude that the TRAIL death pathway is present and can function in thyrocytes, but that a labile inhibitor of the TRAIL death pathway is present in thyrocytes. In addition, TRAIL expression is up-regulated by inflammatory cytokines in thyrocytes, and TRAIL expressed by a papillary thyroid carcinoma cell line was capable of specifically killing a TRAIL-sensitive cell line. These data indicate that the TRAIL-mediated cell death pathway may be important in thyroid disease.

MATERIALS AND METHODS

Cells, Cell Cloning, Cell Culture, and Cytokine Treatments—The KAT5 human papillary thyroid carcinoma-derived cell line (31), BJAB, and SW480, a human colon adenocarcinoma (both obtained from American Type Culture Collection), were maintained in RPMI 1640 medium supplemented with 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies, Inc.) with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT). Normal thyroid tissue was obtained from patients at thyroidectomy from the uninvolved, contralateral lobes of thyroids with tumors. All excised tissues were prepared for cell culture as described previously (32). The primary cultures were passaged in CytoGra Complete media (Mediatech, Herndon, VA) supplemented with 10% NuSerum IV (Collaborative Biomedical Products, Bedford, MA), 100 units/ml penicillin, and 100 μg/ml streptomycin. Primary thyrocytes were cloned by sparse plating after initial digestion, allowing the intact follicles to become adherent and isolating individual follicles with cloning rings by standard techniques. Thyroglobulin staining was performed as described previously (32) to determine thyrocyte purity, and only cultures that were >95% thyroglobulin-positive were used for experiments. Intrathyroidal lymphocytes were isolated from tissue obtained from a patient diagnosed with Hashimoto's thyroiditis, and after two cycles of panning to remove adherent thyrocytes, the nonadherent intrathyroidal lymphocytes were harvested for RNA isolation. Cells were treated with cytokines as described in the figure legends at the following concentrations: IFN-γ, 100 units/ml (Roche Molecular Biochemicals); TNF-α, 50 ng/ml (Collaborative Biomedical Products); IL-1β, 50 units/ml (Sigma Chemical Co.); and TRAIL (affinity-purified as described previously (19) using bacterial expression from plasmid pET15β-His-FLAG-TRAIL).

RNase Protection Assays—RNA was isolated from cells using Trizol reagent according to the manufacturer's protocol (Life Technologies, Inc.). Multiprobe RNase Protection Assay System (PharMingen, San Diego, CA) was used for the detection and quantitation of multiple, specific mRNA species.32P-labeled antisense RNA probes were prepared using the human apoptosis hApO-3 and hApO-3c template sets (PharMingen), which included probes for human TRAIL, DR4, DR5, and GAPDH. This was performed as described previously (32), according to the manufacturer's protocol. Briefly, the probes were hybridized overnight at 2–8°C to 100 μg total RNA from treated and control cultures. After hybridization, the samples were subjected to RNase treatment, followed by purification of RNase-protected probes. The protected probes were resolved on a 5% denaturing polyacrylamide gel. We quantified transcripts by autoradiography, followed by densitometry. Relative amounts of message were corrected for RNA loading by comparison with the GAPDH band intensity for each sample.

Determination of Cell Viability and PCD—Cell viability was determined by MTT assay (33) as described in Ref. 32 and by staining with fluorescein diacetate and propidium iodide (PI) quantitated by flow cytometry as described in Ref. 34. Specific inhibition of TRAIL-induced cell death was performed using TRAIL-neutralizing monoclonal antibody clone M180 (a kind gift from Immunex Corp., Seattle, WA). PCD was determined by visual examination of morphological changes in the cells (membrane blebbing, rounding up, nonadherence). Western blot analysis of PARP cleavage and flow cytometry analysis of DNA fragmentation and annexin V staining were used to confirm that apoptosis had occurred. Western blot analysis was performed as described previously (35) using clone C2-10 anti-PARP monoclonal antibody (PharMingen). Flow cytometry analysis of DNA fragmentation (TUNEL staining) was performed as described previously (34). Briefly, cells were trypsinized and harvested from the plates, washed once with phosphate-buffered saline, and fixed with 1% formaldehyde for 15 min on ice. Nonadherent cells were collected separately and added back to the harvested trypsinized cells before washing with phosphate-buffered saline. Cells were centrifuged and resuspended in phosphate-buffered saline and mixed with ice-cold 70% ethanol (1 volume of cell suspension:10 volumes of ethanol). The cells were stored at –20 °C overnight before bromodeoxyuridine staining. On the day of staining, cells were centrifuged, washed once with rinsing buffer (phosphate-buffered saline supplemented with 0.1% (v/v) Triton X-100 and 5 mg/ml bovine serum albumin). For DNA fragment staining, cell pellets were resuspended and incubated with 25 μl of terminal transferase solution (Roche Molecular Biochemicals) containing reaction buffer, 80 μM bromodeoxyuridine triphosphate, 6.25 units of terminal deoxynucleotidyltransferase, and 1.0 μM CoCl2 for 40 min at 37 °C. The cells were then washed once with rinsing buffer and incubated with 50 μl of fluorescein isothiocyanate-conjugated anti-bromodeoxyuridine monoclonal antibody (Becton Dickinson, San Jose, CA) at room temperature for 1 h. After incubation, cells were resuspended in 1 ml of phosphate-buffered saline containing PI (5 μg/ml) and RNase (200 μg/ml) (Sigma) and incubated for 30 min at room temperature in the dark. Green (anti-bromodeoxyuridine) and red (PI) fluorescence of the cells was determined on a FACSCAN flow cytometer (Becton Dickinson) and analyzed by CellQuest software. Apoptotic cells were defined as having both a euploid DNA content as measured by PI staining and DNA fragmentation greater than control cells as measured by anti-bromodeoxyuridine staining (TUNEL-positive). Dead cells were defined as subdiploid. Other cells (euploid, TUNEL-negative) were considered live. Annexin V staining was performed as described by the manufacturer (Roche Molecular Biochemicals) and analyzed by flow cytometry.

Cell-mediated Cytotoxicity Assay—Cell-mediated cytotoxicity assays were performed using the LIVE/DEAD® Cell-mediated Cytotoxicity Kit (Molecular Probes, Inc., Eugene, OR). Briefly, KAT5 cells were plated at 5.0 × 104 cells/well in a 96-well tissue culture plate and allowed to adhere for 5 h. These cultures were pretreated for 24 h with inflammatory cytokines IFN-γ (100 units/ml) and TNF-α (50 ng/ml) to induce TRAIL expression and washed three times with media to remove cytokines before adding BJAB cells. BJAB cells were labeled with DiOC6 stain as described by the kit manufacturer and combined with KAT5 cultures at 4.0 × 104 cells/well. Control and neutralizing antibodies were added to KAT5 cultures 1 h before adding BJAB cells at a final concentration of 2 μg/ml. PI was added at 50 μl/ml. The cells were co-cultured overnight. Nonadherent cells were removed and saved for analysis by flow cytometry as described by the kit manufacturer.

RESULTS

Primary Thyrocytes and Thyroid Papillary Carcinoma-derived Cell Line KAT5 Express DR4 and DR5—RNase protection assays were used to determine the expression of DR4 and DR5 mRNA in thyrocytes. SW480 cells were used as a positive control. As shown in Fig. 1, four separately isolated, cloned cultures of primary thyrocytes were assayed, and each showed similar levels of expression of DR4 and DR5, respectively. KAT5 cells also expressed DR4 and DR5.

TRAIL Induces Programmed Cell Death in Primary Thyrocyte Cultures—To determine whether TRAIL receptors identified in the thyroid are functional for inducing programmed cell death, we treated primary thyrocytes with purified recombinant TRAIL. Fig. 2A shows that the viability of primary thyrocytes is unchanged by the addition of TRAIL to the cell
culture media as measured by MTT assay. However, because a labile inhibitor of CD95-mediated cell death has been reported to be present in thyrocytes (32), we pretreated cells for 72 h with CHX (10 μg/ml) and then treated with 4-fold dilutions of TRAIL for 16 h compared with control cells treated with CHX only. The assay was performed in the presence of 3.0 μg/ml control IgG1 antibody or TRAIL-neutralizing antibody clone M180. C, a time course of TRAIL (800 ng/ml)-treated thyrocytes after a 72-h CHX pretreatment. Dead cells were determined by flow cytometry after simultaneous staining with fluorescein diacetate and PI. 1.0 × 10^6 cells/sample were analyzed. D, annexin V-fluorescein isothiocyanate staining of thyrocytes treated as described in C and measured by flow cytometry. Only PI-negative cells (>600/sample) were included when calculating the percentage of annexin-V positive cells. E, TUNEL staining of thyrocytes treated with CHX for 67 h followed by incubation with or without 800 ng/ml TRAIL for 5 h was measured by flow cytometry. Only intact cells (>800/sample) were used in the analysis as identified by forward and side scatter.

To definitively prove that the loss of viability was due to PCD, we assayed for phosphatidylserine expression on the outer layer of the plasma membrane by annexin V-fluorescein isothiocyanate staining and for nuclear DNA fragmentation of treated cells by flow cytometry. Fig. 2D shows the results of annexin V-fluorescein isothiocyanate staining of treated, viable cells 2, 4, and 6 h after TRAIL treatment compared with the untreated control. Intact cells (PI-negative) showed an increased percentage of cells with annexin V binding after 4 (23% above control) and 6 h (38% above control) of treatment. TUNEL staining was also performed to determine whether DNA fragmentation, a specific marker for PCD, occurred in these thyrocyte cultures. As displayed in Fig. 2E, 70% of thyrocytes treated with TRAIL for 5 h after CHX pretreatment had become apoptotic (euploid/TUNEL-positive) compared with less than 1% of control CHX-treated cells.

**TRAIL Induces Programmed Cell Death in KAT5 Cells**—The papillary thyroid carcinoma-derived KAT5 cell line was used to confirm TRAIL death-inducing activity in a defined thyroid cell line. KAT5 cells are sensitive to CD95-mediated PCD when combined with CHX (data not shown), and cancer cell lines, in general, are more likely to be susceptible to TRAIL (17). As in the primary thyrocytes, TRAIL alone could not reduce viability in KAT5 cells at a concentration of 800 ng/ml as determined by the MTT assay (Fig. 3A). When combined concomitantly with CHX, TRAIL (6.4 ng/ml) significantly reduced the viability of
KAT5 cells (63% reduction) compared with CHX treatment alone (Fig. 3B). The reduction in viability was almost completely inhibited by a specific TRAIL-neutralizing antibody. Visual observation of the cell cultures after TRAIL and CHX treatment revealed that the KAT5 cells exhibited morphological changes indicative of PCD including membrane blebbing, rounding up, and loss of adherence (data not shown). To further verify this finding, we used Western blot analysis to determine the cleavage of PARP, an indicator of PCD. The Western blot analysis in Fig. 3C shows that treatment of KAT5 with TRAIL and CHX induces specific PARP cleavage from the full-size 116-kDa protein to the diagnostic 85-kDa fragment within 6 h, thus confirming that PCD is occurring in these cells.

**Fig. 3.** KAT5 cells are susceptible to TRAIL-induced PCD in the presence of CHX. A, relative viability of KAT5 cells treated for 20 h with TRAIL (800 ng/ml) compared with untreated cells, as measured by the MTT assay. B, relative viability of KAT5 cells treated for 20 h with TRAIL at various dilutions and CHX (10 μg/ml) compared with control cells treated with CHX only. The assay was performed in the presence of 0.3 μg/ml control antibody or TRAIL-neutralizing antibody. C, Western analysis of PARP cleavage in KAT5 cells treated with TRAIL (800 ng/ml) and CHX (lane 1) compared with CHX-treated controls (lane 2). Cells were treated for 6 h before harvesting cell lysate.

KAT5 cells (63% reduction) compared with CHX treatment alone (Fig. 3B). The reduction in viability was almost completely inhibited by a specific TRAIL-neutralizing antibody. Visual observation of the cell cultures after TRAIL and CHX treatment revealed that the KAT5 cells exhibited morphological changes indicative of PCD including membrane blebbing, rounding up, and loss of adherence (data not shown). To further verify this finding, we used Western blot analysis to determine the cleavage of PARP, an indicator of PCD. The Western blot analysis in Fig. 3C shows that treatment of KAT5 with TRAIL and CHX induces specific PARP cleavage from the full-size 116-kDa protein to the diagnostic 85-kDa fragment within 6 h, thus confirming that PCD is occurring in these cells.

**TRAIL mRNA Is Induced by Inflammatory Cytokines in Thyrocytes**—RNA protection assays were performed on total RNA isolated from cloned, normal primary thyrocytes, KAT5 cells, and intrathyroidal lymphocytes derived from a Hashimoto’s thyroiditis gland sample to determine whether there was an endogenous production of TRAIL in the thyroid. These cells were treated for 6 h with various types of inflammatory cytokines before harvest and RNA isolation. RNA protection assays revealed that inflammatory cytokines can up-regulate expression of TRAIL mRNA in normal thyrocytes and KAT5 cells. TRAIL mRNA expression significantly increases in response to IFN-γ treatment in both normal thyrocytes and KAT5 cells and shows smaller increases induced by TNF-α and IL-1β treatment in normal thyrocytes. KAT5 cells also show TRAIL mRNA induction by TNF-α but not by IL-1β. The combination of IFN-γ with either TNF-α or IL-1β treatment synergized to produce dramatic increases in TRAIL message in both normal thyrocytes and KAT5 cells. The TRAIL mRNA expression pattern in response to cytokine treatment of the normal thyrocytes shown in Fig. 4 is representative of the results observed with six different patient samples. TRAIL mRNA was also expressed in unstimulated intrathyroidal lymphocytes isolated from a patient with thyroiditis. This suggests that TRAIL may be present within the inflamed thyroid.

**Inflammatory Cytokine-treated KAT5 Cells Kill BJAB Cells through a TRAIL-dependent Mechanism**—To determine whether TRAIL expressed by thyroid-derived cells is functional, we performed a cell-mediated cytotoxicity assay. We combined KAT5 cells with TRAIL-sensitive BJAB cells and measured BJAB cell death. Fig. 5 shows that KAT5 cells pretreated with the combination of cytokines that induced the greatest expression of TRAIL (IFN-γ and TNF-α) are capable of inducing cell death in >40% of BJAB cells compared with less than 10% cell death by untreated KAT5 cells. TRAIL-neutralizing antibody was capable of inhibiting BJAB killing by cytokine-treated KAT5 cells, reducing cell death to 25%. A Fas-neutralizing antibody was completely ineffective in inhibiting BJAB cell death. We also obtained similar results with a primary papillary thyroid cancer-derived culture.

**DISCUSSION**

We have demonstrated that DR4 and DR5 mRNA are expressed in normal primary thyrocytes and in the papillary thyroid cancer-derived cell line KAT5. Primary thyrocytes and KAT5 cells were found to be susceptible to PCD induced by TRAIL, the ligand for DR4 and DR5, in the presence of protein

**Fig. 4.** TRAIL is expressed in inflammatory cytokine-treated thyrocytes and KAT5 cells. RNase protection assays show TRAIL induction in thyrocytes (A) and KAT5 cells (B) treated for 6 h with inflammatory cytokines. GAPDH is included as a control for relative RNA levels. An underexposure of the GAPDH bands is also included to more clearly demonstrate the relative RNA levels.
Fig. 5. **Inflammatory cytokine-treated KAT5 cells kill TRAIL-sensitive BJAB cells by a TRAIL-dependent mechanism.** After pretreatment with or without inflammatory cytokines, KAT5 (effector) cells were co-incubated with BJAB (target) cells in the presence of isotype-matched control IgG1, TRAIL-neutralizing antibody (clone M180), or Fas-neutralizing antibody (clone ZB4). BJAB cells alone were incubated with or without Fas agonist (clone CH11) and/or neutralizing (clone ZB4) antibody as controls. Dead BJAB cells were measured by flow cytometry as described under “Material and Methods.” The data are the means ± S. D. of triplicate determinations with a significance set at a 95% confidence level.

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by phosphatidylinositol-specific phospholipase C did not alter the TRAIL sensitivity of KAT5 cells (data not shown). Determination of the inhibitor(s) of the TRAIL death pathway in thyrocytes is of value because it may suggest possible mechanisms of pharmacological intervention in PCD-influenced thyroid disease.

Recently, PCD signaling through the TRAIL-related CD95I-CD95 ligand/receptor pair has received attention as an explanation for autoimmune thyrocyte destruction (29, 46, 47). Data by Giordano et al. (29) suggest that thyrocytes may commit suicide when IL-1β up-regulation of CD95 allows constitutively expressed CD95L to induce PCD in thyrocytes. We have been unable to document CD95L expression in thyrocytes (30), but this does not preclude the possibility that TRAIL may be acting through its death receptors in a manner postulated for CD95L by Giordano et al. (29).

Our data suggest three possible nonexclusive scenarios for the involvement of thyrocyte-derived TRAIL in the etiology of thyroid diseases. The first scenario is that inflammatory cytokine-induced thyrocyte-expressed TRAIL could initiate thyrocyte destruction symptomatic of chronic autoimmune thyroiditis. This mechanism is similar to that proposed for CD95L as described above (29, 47); however, the fact that this pathway is blocked argues against this. The second scenario is that inflammatory cytokine-induced thyrocyte-expressed TRAIL could mediate thyrocyte evasion of autoimmune attack by killing the invading, cytokine-expressing lymphocytes. This would create a site of immune privilege within the thyroid. The third scenario is that inflammatory cytokine-induced thyroid carcinoma-expressed TRAIL could mediate tumor evasion of the immune system by killing the invading, cytokine-expressing lymphocytes. Supporting this scenario is our data that confirm that inflammatory cytokine-induced-trial expression by papillary thyroid carcinoma cell line KAT5 is functional in killing the lymphoid cell line BJAB through a TRAIL-dependent mechanism. In addition, supporting the latter two scenarios are the findings that activated T lymphocytes are known to be susceptible to TRAIL-induced cell death (14–16), and IFN-γ-treated thyrocytes are resistant to autologous cytotoxic T lymphocyte- and natural killer-mediated cytotoxicity (48). Also, there is evidence to suggest that papillary thyroid carcinomas are capable of evading immune attack in some cases (49–51). If normal thyroid follicular cells lost the capacity to produce TRAIL, they might lose the ability to fight off an autoimmune attack.

In summary, we have demonstrated that DR4 and DR5 are expressed, and their ligand, TRAIL, can mediate PCD in thyrocytes. In addition, thyrocytes themselves can be induced to
express functional TRAIL capable of killing lymphoid cells. This raises many possibilities for thyroid disease involvement by TRAIL. A greater understanding of the regulation and function of the TRAIL death pathway in the thyroid, including the determination of its endogenous inhibitors, may therefore provide insights into the factors involved in thyroiditis-induced thyroid damage and thyroid cancer.

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