Expression of TNF-related apoptosis inducing ligand (TRAIL) on infiltrating cells and of TRAIL receptors on salivary glands in patients with Sjögren's syndrome

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Abstract

Background

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) induces apoptosis of tumor cells but not normal cells; its role in normal non-transformed tissues is unknown.

Objective

To evaluate the role of apoptosis mediated by TRAIL and TRAIL-receptor (TRAIL-R) system in lymphocytic sialadenitis in patients with Sjögren's syndrome.

Methods

The expression of TRAIL and TRAIL-R1, 2, 3 and 4 in lymphocytic sialadenitis was examined by immunoperoxidase staining in patients with Sjögren's syndrome and in normal subjects. To elucidate the mechanism of de novo expression of TRAIL-R1 antigen, we quantitatively investigated its induction by cytokines in human salivary duct cell line (HSG) by cell enzyme-linked immunosorbent assay. In human salivary duct cells stimulated by cytokines, we investigated the induction of apoptotic cell death by recombinant TRAIL protein.

Results

In patients with massive mononuclear cell infiltration, some infiltrating cells showed TRAIL. In patients with severe lymphocytic sialadenitis, TRAIL-R1, TRAIL-R2, or both were strongly expressed on the ductal epithelial cells. Neither TRAIL-R3 nor R4 were observed on ductal epithelium. In contrast, TRAIL-R1 and R2 were not found in the minor salivary glands of normal subjects or patients with mild lymphocytic sialadenitis. Unstimulated HSG cells did not express TRAIL-R1. Interferon-γ (IFN-γ) consistently upregulated levels of TRAIL-R1. In contrast, tumor necrosis factor-α (TNF-α), interleukin 1-β (IL-1β), IL-2, and IL-4 had no effect on TRAIL-R1 levels. HSG cells expressing TRAIL-R1 in response to IFN-γ were susceptible to apoptosis by recombinant TRAIL protein.

Conclusion

Our findings suggest that TRAIL and TRAIL-R system may play a role in the pathogenesis of lymphocytic sialadenitis in patients with Sjögren's syndrome.

Key words

Sjögren's syndrome, tumor necrosis factor-related apoptosis-inducing ligand, apoptosis, lymphocytic sialadenitis.

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Introduction

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is a recently identified type ll integral membrane protein belonging to the TNF family, which induces apoptotic cell death in a variety of tumor cells (1-3). Among members of this family, TRAIL exhibits high homology to Fas L/CD95L, which has been implicated in T-cell cytotoxicity and immune regulation (4). TRAIL mRNA is found in a variety of tissues and cells (1), but its physiological role remains unknown.

Recent studies have revealed that at least four receptors, (TRAIL-R1/DR4, TRAIL-R2/DR5/TRICK2, TRAIL-R3/TRID/DcR1/LIT, and TRAIL-R4/TRUNDD/DcR2) bind to TRAIL with similar affinities (5-10). Both TRAIL-R1 and TRAIL-R2 contain a death domain homologous to that in Fas, TNFR-1, and DR3. Oligomerization of the death domain in TRAIL-R1 and TRAIL-R2 recruits caspase-8 or -10 via Fas-associated death domain (FADD) or FADD-like adapter molecules and activates the subsequent caspase cascade, resulting in apoptotic cell death (5-8, 10). In contrast to these receptors, TRAIL-R3 lacks a cytoplasmic domain. Because TRAIL-R3 can compete with the proapoptotic TRAIL-receptors (TRAIL-Rs) for TRAIL binding, it seems to work as a decoy receptor. TRAIL-R4 carries a cytoplasmic domain containing a truncated death domain that cannot transmit a death signal but can activate nuclear factor kB, which may protect the cells from TRAIL-R1- and TRAIL-R2-induced apoptosis (9, 10).

In vivo administration of recombinant TRAIL selectively kills tumor cells but not normal cells, leaving host organ systems unharmed (11,12). Fundamental questions regarding the role of TRAIL in normal nontransformed tissues have thus been raised. Recently, Song *et al.* found that administration of recombinant soluble TRAIL-R2 blocks autoimmune arthritis in mice with collagen-induced arthritis (13). They concluded that TRAIL is a potent inhibitor of autoimmune arthritis and that this effect may result from inhibition of cell cycle progression, cytokine production or both.

Sjögren's syndrome (SS) is a chronic autoimmune disorder characterized by dysfunction and destruction of the salivary, lacrimal, and other exocrine glands. Histologically, marked infiltration of lymphoid cells is commonly found in the lacrimal and salivary glands. The majority of infiltrating lymphocytes are activated CD4+ T cells (14, 15) and produce cytokines such as interleukin-2 (IL-2) and interferon-γ (IFN-γ) (16-19). We previously reported that in lymphocytic sialadenitis of SS, *de novo* expression of Fas antigen was observed on the epithelial cells of the minor salivary ducts, especially in cases with severe mononuclear cell infiltration, and that several infiltrating cells showed Fas ligand around the minor salivary ducts (20-22). Staining with DNA nick end labeling methods showed that ductal epithelial cells undergo by apoptosis (21, 23). This finding suggests that the Fas/Fas ligand system may play a role in the pathogenesis of lymphocytic sialadenitis in Sjögren's syndrome by providing a specific target for cytotoxic T-cells expressing Fas ligand. Cross-linking of Fas antigen on ductal epithelium to Fas ligand on infiltrating lymphocytes may thereby induce apoptosis in ductal epithelial cells (24). Although TRAIL exhibits high homology to Fas ligand and is thought to play a role in T-cell cytotoxicity, the role of TRAIL in lymphocytic sialadenitis of SS remains unknown. We therefore studied the expression of TRAIL and TRAIL-R in lymphocytic sialadenitis in patients with SS and the expression and roles of TRAIL-R on human salivary ductal cells.

Materials and methods

The minor salivary glands of 19 patients (17 women, 2 men, age 22 to 80 years; mean 49.4) with SS were studied. All patients had sicca symptoms and showed characteristic lymphocytic infiltrate in specimens of salivary glands obtained by lip biopsy. All patients fulfilled the criteria for the European classification criteria for Sjogren's syndrome. Three patients fulfilling the criteria for systemic lupus erythematosus, 2 with mixed connective tissue dis-

ease and one fulfilling the criteria for rheumatoid arthritis were regarded to have secondary SS. The other 13 patients were given a diagnosis of primary SS. For normal controls, three lip biopsy specimens were studied as the same way. Those normal minor salivary glands were obtained for diagnostic purposes from patients with fever of unknown origin, but no abnormal changes were found. All of the patients agreed to the investigation and gave their signed informed consent.

Immunoperoxidase staining

Samples of minor salivary glands were obtained by lip biopsies. Tissue sections were prepared for immunoperoxidase staining as described elsewhere. To detect TRAIL, first antibody was monoclonal antibody to human TRAIL (RIK-1, a gift from Dr Kayagaki N and Dr Okumura K) (25) and second antibody was Envision solution+® (goat anti-mouse immunoglobulins conjugated to peroxidase labelled-dextran polymer; Dako Japan, Kyoto, Japan). To detect TRAIL-R1, first antibody was polyclonal antibody to human TRAIL-R1(Pharmingen, Fujisawa, Japan), second antibody was Envision solution+ rabbit® (goat anti-rabbit immunoglobulins conjugated to peroxidase labeled dextran polymer; Dako Japan). To stain TRAIL R2, 3, and 4, first antibodies were polyclonal antibody to human TRAIL-R2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), TRAIL-R3, and TRAIL-R4 (R&D systems, Minneapolis, MN, USA) respectively, second antibodies were streptaviotin-labeled rabbit anti-gout immunoglobulins (Dako Japan), biotin-peroxidase (Dako Japan), and 0.02% 3,3`-diaminobenzidine (DAB) containing 0.03% H_2O_2 and 10 mM sodium azide.

A surgically removed lymph node showing non-specific chronic lymphadenitis and human TRAIL transfected BHK cells (25) were used as a positive control. The following negative controls were used: (a) omission of primary antibody or (b) appropriately diluted mouse, rabbits or goat immunoglobulins as a first layer. In all negative controls, only a few granulocytes showed endogenous peroxidase activity.

Histologic grading

To assess inflammation in the salivary gland biopsy specimens, the number of focal aggregates including at least 50 mononuclear cells per 4 mm^2 was counted (26). We graded ductal TRAIL-R1 or R2 expression according to the following criteria: no TRAIL-R1 or R2 expression, grade 0; TRAIL-R1 or R2 expression by a few ductal cells, grade 1; and TRAIL-R1 or R2 expression by all cells of one duct or by many ductal cells, grade 2.

Tissue culture and treatment of cells with cytokines

HSG cells (a gift from Dr Mitsunobu Sato, Tokushima University, Japan) were cultured in HAM F-12 Medium (Life Technologies, NY, USA) supplemented with 10% fetal calf serum (Biowhiffaker MD, USA) at 37°C with 5% CO_2.

Recombinant human IFN-γ (R &D Systems), recombinant human TNF-α (Pharmingen, San Diego, CA, USA), recombinant human IL-1β (R & D Systems), human IL-2 (Roche Diagnostics GmbHs Mannheim, Germany), and recombinant human IL-4 (Genzyme, Cambridge, MA,USA) were used. Cells were cultured in the presence of IFN-γ, 1 to 100 U/ml; TNF-α, 1 to 100 u/ml; IL-1β, 0.01 to 1 ng/ml; IL-2, 0.02 to 2 U/ ml; or IL-4, 0.002 to 0.2 ng/ml, for 6 to 48 hours.

Cell enzyme-linked immunosorbent assay (ELISA)

A total of 8 x10^5 cells/well were seeded onto gelatin-coated, 96-well, flat-bottomed microtiter plates (Falcon 3072, Becton Dickinson, Ruhterfold, NJ, USA) in 0.2 ml of medium. After reaching confluence (~48 hr), the culture medium was replaced with 0.2 ml of fresh medium containing various concentrations of cytokines and was incubated for various periods of time at 37°C in 5% CO_2. The cultures were fixed in 3% paraformaldehyde in phosphate buffered saline (PBS) containing 8% sucrose for 30 min at room temperature. Subsequent washing steps were performed with five changes of PBS with 0.05% Tween 20® (Wako, Osaka, Japan). To avoid nonspecific binding of antibodies, the assays were performed after pretreatment with PBS and 50% Blockace® (Yukizirushi, Sapporo, Japan). After washing, the fixed monolayer was incubated with 0.1 ml/well of 5% normal goat serum PBS for 1 hour. The blocking solution was added with 500 ng of mouse monoclonal antibody to TRAIL-R1 (Pharmingen) and the mixture was incubated for 120 min at 37°C. After washing, the plates were treated with 0.1 ml/well of Envision solution+ rabbit® for 75 min at room temperature. The plates were washed again and incubated with 0.1 ml of TMB+ substrate chromogen (Dako Japan) for 15 to 60 min at room temperature. The plates were read on a microtitier plate reader (SLT Labinstruments, Salzburg, Austria) at 650 nm. All values are expressed as means ± SD of triplicate wells. For the dose-dependent, time-course, and blocking experiments, the data are reported as optical density (OD) achieved in a given experiment (mean ± SD of triplicate wells) after subtracting the background OD of the wells for negative control. As positive control for the cell ELISA system, epithelial membrane antibody (EMA, Dako Japan) was used. The following negative controls were used: (a) omission of primary antibody or (b) appropriately diluted mouse IgG as a first layer.

Specificity examination of IFN-γ stimulation

To examine the specificity of IFN-γ, we tested the ability of antibody to IFN-γ to block the expression of TRAIL-R1. Pretreatment of IFN-γ with anti-IFN-γ antibody for 30 min before stimulation with IFN-γ resulted in elimination of the upregulation of TRAIL-R1 expression

Induction of apoptotic cell death by recombinant TRAIL

A total of 8 x 10^5 cells/well were seeded onto 96-well, flat-bottomed microtiter plates (Falcon 3072, Becton Deckinson, NJ, USA) in 0.2 ml of medium. After reaching confluence (~48 hr), the culture medium was replaced with 0.2 ml of fresh medium containing IFN-γ and was incubated for 20 hrs at 37°C in 5% CO_2. After washing, the plates were treated with 5 x 10^{-6} to 5 x 10^{-4} mg/ml

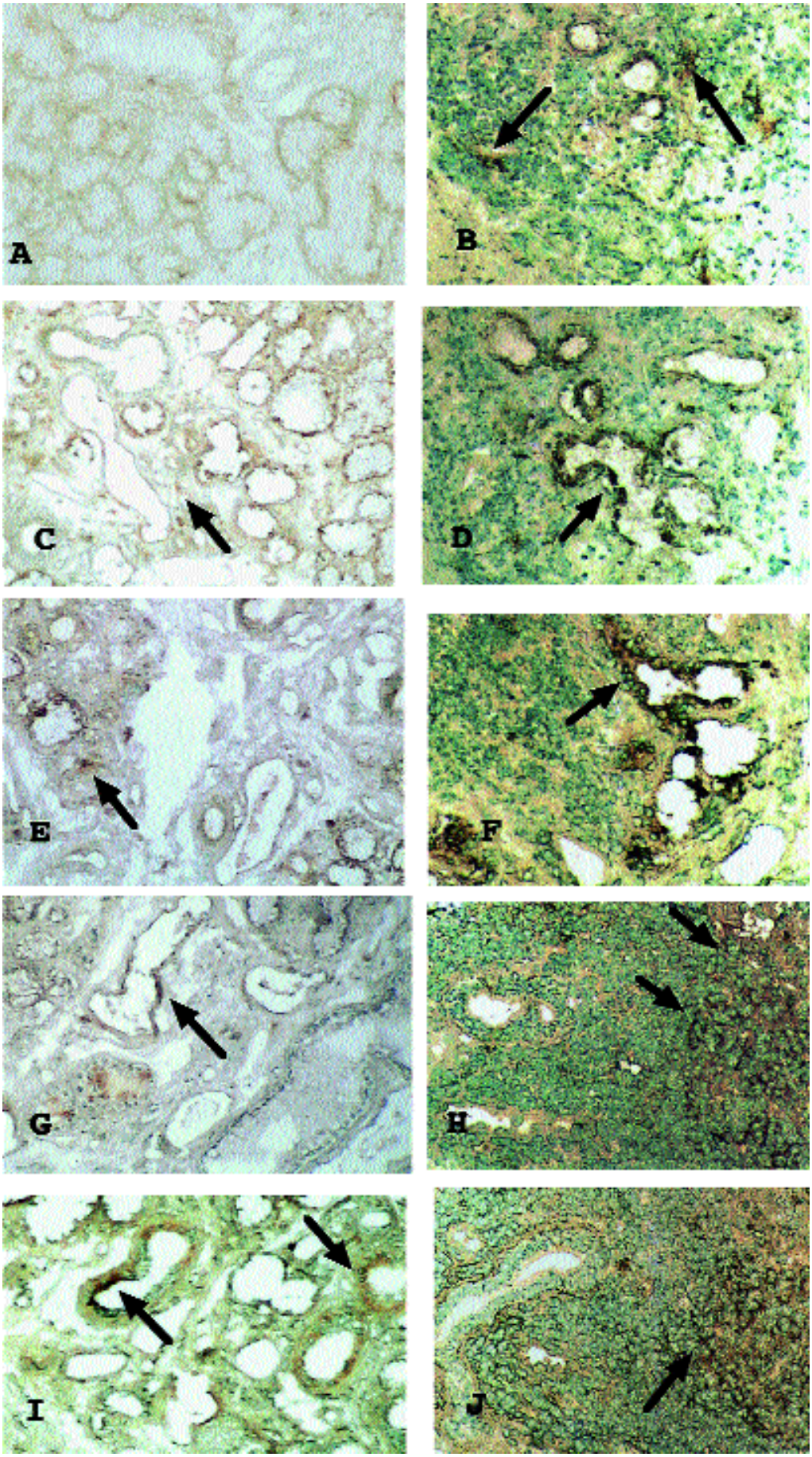

Fig. 1. Immunohistochemical staining of TRAIL and TRAIL-Rs in salivary gland.
(A) TRAIL was not observed in normal control (x200); **(B)** TRAIL was observed on many infiltrating mononuclear cells in severe lymphocytic sialadenitis around the duct (x180); **(C)** TRAIL-R1 was not observed on either acinar or ductal cell in normal control. Some interstitial cells showed TRAIL-R1 (arrow) (x200); **(D)** TRAIL-R1 on ductal epithelial cells (arrow) and some infiltrating mononuclear cells in severe lymphocytic sialadenitis (x180); **(E)** TRAIL-R2 was not observed on either acinar or ductal cells in normal control. Some interstitial cells showed TRAIL-R2 (arrow) (x180); **(F)** TRAIL-R2 was expressed on ductal epithelial cells (arrow) and some infiltrating mononuclear cells in severe lymphocytic sialadenitis (x180); **(G)** In normal control, some ductal epithelial cells showed TRAIL-R3 (arrow) (x180); **(H)** In severe lymphocytic sialadenitis, TRAIL-R3 was not observed on ductal epithelial cells. Many infiltrating mononuclear cells showed TRAIL-R3 (arrow) (x200); **(I)** In normal control, some ductal epithelial cells showed TRAIL-R4 (arrow) (x200); **(J)** In severe lymphocytic sialadenitis, TRAIL-R4 was not observed on ductal epithelial cells. Many infiltrating mononuclear cells showed TRAIL-R4 (arrow) (x200)

of recombinant human TRAIL (R &D Systems) conjugated by anti-histidine antibody (R &D Systems) for 6 hours. Dead cells were counted by trypan blue dye exclusion methods.

Statistical analysis

Cell ELISA data were analyzed with Student's t-test.

Results

TRAIL and TRAIL-R expression on salivary glands in patients with SS

No TRAIL-positive cells were observed in normal salivary glands (Fig. 1A). TRAIL was seen on some infiltrating mononuclear cells of the minor salivary glands in all patients with SS (Fig. 1B). TRAIL-R1 and R2 were not expressed on either acinar cells or ductal cells in normal salivary glands (Fig. 1C, E). TRAIL-R1 and TRAIL-R2 were expressed on some interstitial cells in all patients with SS. In patients with massive mononuclear cell infiltration, TRAIL-R1 and R2 were expressed on ductal epithelial cells and a few acinar epithelial cells in the lip biopsy specimens (Fig. 1D, F). Ductal TRAIL-R1 staining was observed in 13 cases of 19 SS patients. TRAIL-R2 expression on ductal epithelial cells was observed 17 cases of 19 SS patients.

Several TRAIL-R3-or R4-positive cells were observed in salivary gland ducts in normal salivary glands (Fig. 1G, I). TRAIL-R3 and R4 were not seen on ductal epithelium in area of massive cellular infiltration and many infiltrating mononuclear cells showed TRAIL-R3 and R4 (Fig. 1 H, J).

Ductal TRAIL-R1 and R2 expression positively correlated with the grade of inflammation of the salivary glands (Fig. 2 A, B). The differences between primary SS and secondary SS was not observed.

Expression of TRAIL-R1 molecules in HSG cell line

Using an immunoperoxidase-cell ELISA, we found that unstimulated HSG cells constitutively expressed low levels of TRAIL-R1 molecule. IFN-γ (1 to 100 U/ml) consistently upregulated constitutively expressed levels of TRAIL-R1 in a dose-dependent fashion (Fig. 3A). The time courses of IFN-γ-induced TRAIL-R1 expression are shown in Figure 3B. A significant increase in TRAIL-R1 expression was observed 12 to 24 hours after the addition of IFN-γ (100 U/ml).

To examine the specificity of IFN-γ, we tested the ability of antibody to IFN-γ to block the expression of TRAIL-R1. Pretreatment of IFN-γ with anti-IFN-γ antibody for 30 min before stimulation resulted in elimination of the upregulation of TRAIL-R1 expression (Fig. 3A).

Six to 48 hours of incubation with TNF-α, IL-1β, IL-2, or IL-4 did not influence the expression of TRAIL-R1 on HSG cells (data not shown).

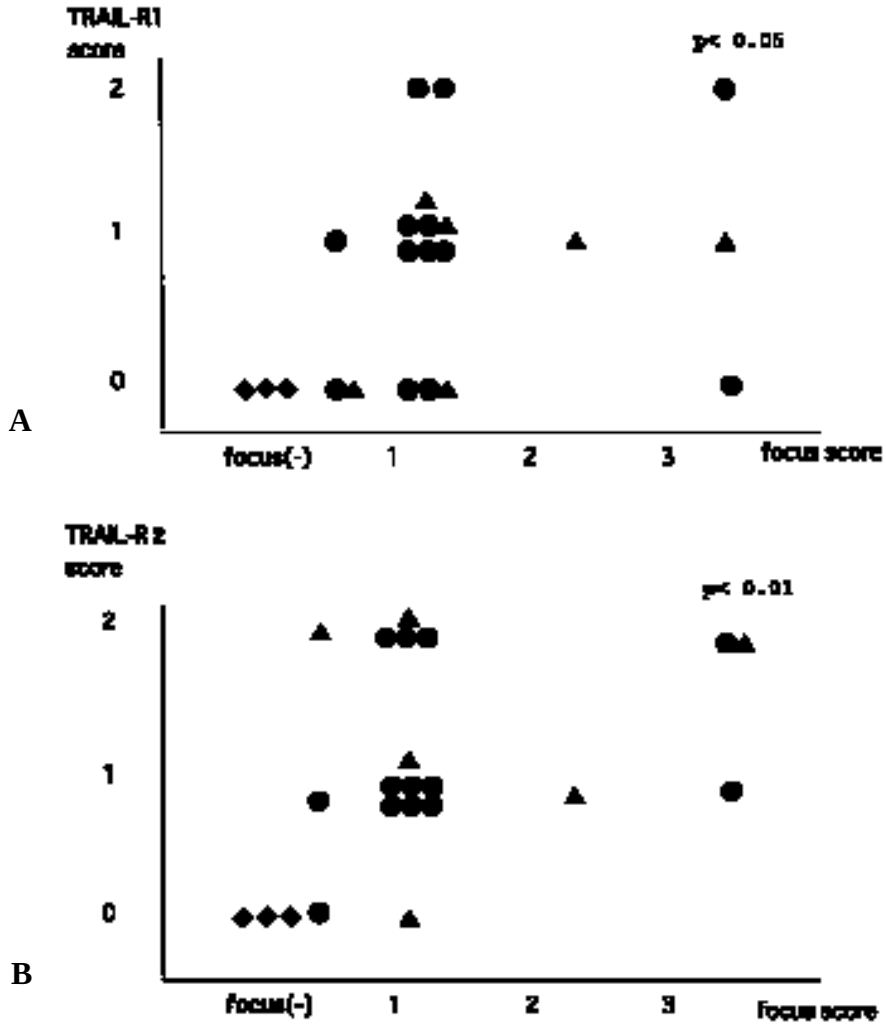

Fig. 2. Ductal TRAIL-R1 **(A)** and TRAIL-R2 **(B)** expression and salivary gland lymphocytic infiltration. Ductal TRAIL-R1 and TRAIL-R2 expression was demonstrated by grading scale. TRAIL-R1 and TRAIL-R2 expression of ductal cells were correlated with interstitial infiltration. (TRAIL-R1 $p < 0.05$, TRAIL-R2 $p < 0.01$ in Spearman rank correlation) patients with primary SS; patients with secondly SS; normal controls.

Recombinant TRAIL-induced apoptosis in HSG cell line after stimulation with IFN-γ

Apoptotic cell death was detected by trypan blue dye exclusion methods. Spontaneous cell death was observed in 0.3% of HSG cells after reaching confluence. After stimulation with 100 μ/ml of IFN-γ, treatment with recombinant TRAIL trimer increased the percentage of apoptotic cells in a dose-dependent fashion. At 5×10^{-5} mg/ml or higher concentration of recombinant TRAIL, the percentages of apoptotic cells were significantly higher than those of untreated HSG cells (Fig. 4). Stimulation of 10 u/ml of IFN-γ did not induce apoptotic cell death of HSG

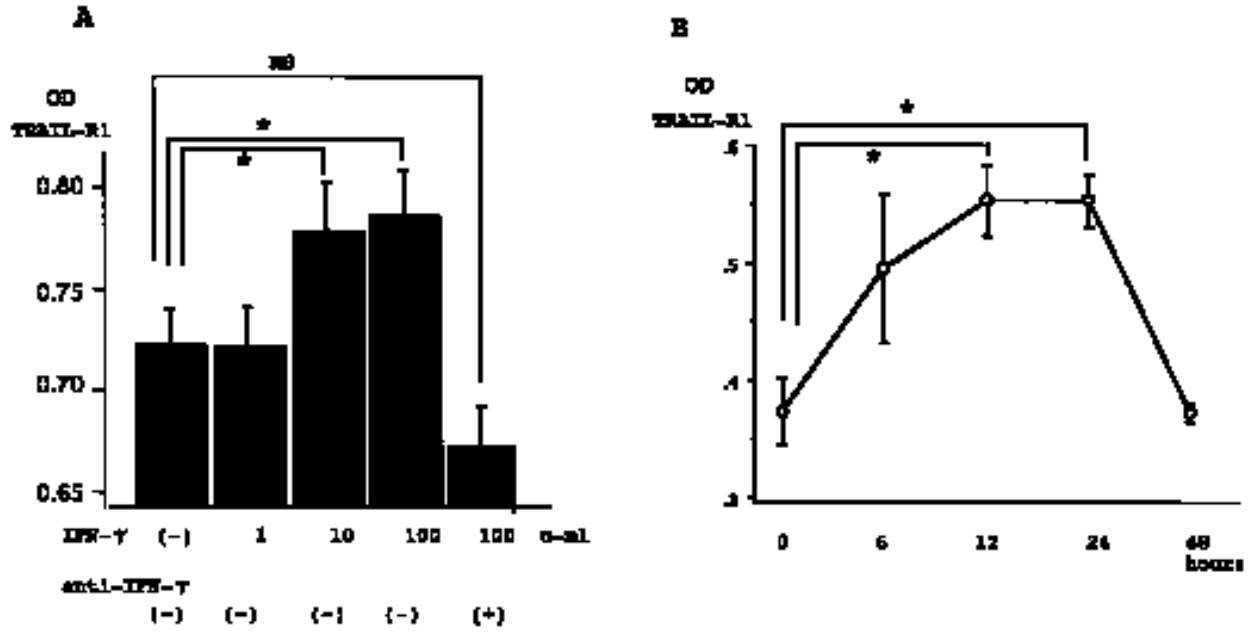

Fig. 3. Effect of IFN-γ on HSG TRAIL-R1 expression as measured by immunoperoxidase cell-ELISA. Cells were cultured and stimulated with IFN-γ. (**A**) TRAIL-R1 expression after 24-hrs' exposure to increasing concentration of IFN-γ and effect of blockade of expression by anti-IFN-γ antibody. HSG cells were cultured and stimulated with 1-100 U/ml of IFN-γ and with 1 μg/ml of monoclonal anti-IFN-γ antibody. Addition of 1 μg/ml of anti-IFN-γ inhibited the upregulation of IFN-γ. (**B**) Time course of induction of TRAIL-R1 by IFN-γ (100 U/ml). All values are expressed as means ± SD of triplicate wells. (*) indicates that the value was significantly different ($p < 0.05$). NS: non-significant. Similar results were obtained in three independent experiments.

cells at 5 x 10^{-4} mg/ml of recombinant TRAIL (data not shown).

Discussion

Cytotoxic T lymphocytes (CTLs) can both specifically recognize and lyse their targets. The well-known perforin-granzyne-based mechanism does not account for all instances of CTL lysis. Recently, the antigen-specific cytotoxicity expressed by cytotoxic T-cells was shown to be Fas based (27, 28). In addition to these two pathways, however, previous studies have suggested other effecter mechanisms for CTL cytotoxicity. For example, Braun et al demonstrated that CTLs from mice deficient in both Fas L and perforin lysed some kinds of tumor cells in an *in vitro* assay (29). Recently, human CD4 cell lines were reported to have perforin-independent and Fas L-independent cytotoxicity against certain target cells that are susceptible to TRAIL-mediated cytotoxicity (25). Such cytotoxic activities were blocked by anti-TRAIL monoclonal antibodies. These results indicate that TRAIL constitutes an additional pathway of T cell-mediated cytotoxicity.

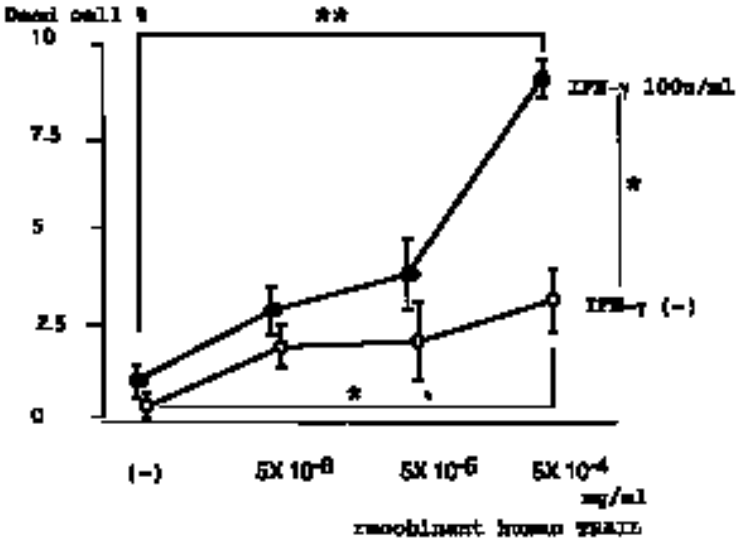

Fig. 4. Induction of apoptotic cell death by recombinant TRAIL.
Recombinant TRAIL trimer induced apoptotic cell death in HSG cells in dose-dependent fashion without IFN-γ stimulation ( ). IFN-γ stimulation ( ) increased HSG cell death induced by TRAIL protein. All values are expressed as means ± SD of triplicate wells. (*) indicates that the value was significantly different ($p < 0.05$). (**) indicates that the value was significantly different ($p < 0.01$). Similar results were obtained in two independent experiments.

The results of our study indicate that TRAIL and TRAIL-R1 and 2 are expressed in salivary glands in patients with SS. In lymphocytic sialadenitis of SS, de novo expression of TRAIL-R1 or R2, which can induce apoptotic cell death, was observed on epithelial cells of the minor salivary ducts, especially in cases with severe mononuclear cell infiltration. TRAIL-R3 and R4, which cannot induce apoptosis, were seen on ductal epithelial cells in normal salivary glands. TRAIL-R3 and R4 were

not observed on ductal epithelium in severe case of lymphocytic sialadenitis associated with SS. Some infiltrating cells showed TRAIL around the minor salivary ducts. Several studies using DNA nick end labeling methods have demonstrated that ductal epithelial cells die by apoptosis (21, 22). This finding suggests that the TRAIL/ TRAIL-R system may play a role in the pathogenesis of lymphocytic sialadenitis in Sjögren's syndrome by providing a specific target for cytotoxic T cells expressing TRAIL.

A variety of methods, including FACS and radioimmunoassay, have been used to measure antigen expression. The cell ELISA was compared with the immunofluoresence staining by Grunow *et al.* (30) and both assays were found to be similarly sensitive. Those results underline the application of the cell ELISA for antigen detection on homogeneous cell lines. We used this procedure to quantify the relative levels of TRAIL-R1 expression in the HSG cell line. In contrast to FACS and radioimmunoassay, our approach avoids mechanical disruption of cell monolayers, trypsinizaton, and the use of radioactivity (30, 31).

A human salivary gland duct cell line (HSG) was established from an irradiated human submandibular salivary gland (32). It has characteristics of intercalated duct cells. HSG cells can mimic several features of SS disease in vivo (33). Cytokines such as IFN-γ and TNF-α induce increased expression of ICAM-1and HLA-DR in HSG cell line. Our study shows that HSG cells, human salivary intercalated duct cells transformed by irradiation, express TRAIL-R1 after IFN-γ treatment and undergo apoptotic cell death in response to TRAIL. In lymphocytic sialadenitis in patients with SS, the majority of infiltrating lymphocytes produce cytokines such as IL-2, IFN-γ, TNF-α, and IL1-β (16-18). IFN-γ secreted by infiltrating activated lymphocytes in sialadenitis associated with SS can stimulate ductal epithelial cells to express TRAIL-R1. HSG cells expressing TRAIL-R1 after IFN-γ stimulation are susceptible to apoptotic cell death by recombinant TRAIL. In lymphocytic sialadenitis associated with SS, ductal epithelial cells expressing TRAIL-R1 may be susceptible to apoptosis by TRAIL on infiltrating lymphocytes. These results can hopefully serve as the basis for cellular models for future investigations addressing the regulation of TRAIL/TRAIL-R expression and apoptosis in SS.

Infiltrating mononuclear cells in lymphocytic sialadenitis in patients with SS showed TRAIL-R3 and R4 as well as R1 and R2. These infiltrating cells are thought to escape from apoptotic cell death because of decoy receptors such as TRAIL-R3 and R4. Surviving lymphocytes may activate and secrete cytokines and then induce tissue destruction. Differential sensitivity to TRAIL is thought to depend on not only on the form of TRAIL-Rs but also on the expression of apoptosis inhibitors. TRAIL-resistant melanoma cell lines contain high levels of FADD-like IL-β-converting enzyme (FLICE)-like inhibitory protein (FLIP) (34). The levels of apoptosis inhibitors such as FLIP in lymphocytic sialadenitis in patients with SS remain unknown. The destruction of ductal epithelium and the survival of infiltrating mononuclear cells may be regulated by a balance among the TRAIL/TRAIL-R system, apoptosis inhibitors, and Fas and bcl-2 in patients with SS. Modification of this balance might induce the improvement or the worsening of SS. Our results emphasize the necessity for further studies of the roles of apoptosis and apoptosis-regulating molecules in the pathogenesis of Sjögren's syndrome and other autoimmune diseases.

Acknowledgment

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