Interferon gamma and tumor necrosis factor alpha induce Fas expression and anti-Fas mediated apoptosis in a salivary ductal cell line


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Abstract

Background

We previously reported that Fas antigen was strongly expressed on salivary duct epithelial cells and that some salivary infiltrating cells showed the Fas ligand in patients with severe sialoadenitis due to Sjögren’s syndrome (SS). Apoptotic changes were observed in ductal epithelial cells and some infiltrating cells by DNA nick end labeling methods. These findings suggest that the Fas-Fas ligand system may play a role in the pathogenesis of sialoadenitis in SS.

Objective

To elucidate the mechanism of the de novo expression of ductal Fas antigen in sialoadenitis associated with SS, we investigated the induction of Fas antigen and apoptosis by cytokines in a human salivary duct cell line.

Methods

Human salivary duct cell line (HSG) was cultured with interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), interleukin 2 (IL-2), interleukin 4 (IL-4), and granulocyte monocyte colony stimulating factor (GM-CSF). The expression of Fas antigen in HSG was examined by immunoperoxidase cell ELISA. The appearance of DNA strand breaks during apoptosis induced by anti-Fas antibody was detected by DNA nick end labeling methods.

Results

Unstimulated HSG cells constitutively expressed low levels of Fas antigen. IFN-γ and TNF-α consistently upregulated constitutive levels of Fas. In contrast, IL-1β, IL-2, IL-4, and GM-CSF had no effect on Fas levels. HSG cells expressing Fas antigen in response to IFN-γ or TNF-α were susceptible to apoptosis by anti-Fas antibody.

Conclusion

Our findings suggest that IFN-γ or TNF-α secreted by infiltrating lymphocytes induces ductal Fas expression and ductal apoptosis in sialoadenitis associated with SS.

Key words

Fas antigen, apoptosis, Sjögren’s syndrome, sialoadenitis, salivary duct cell line, interferon gamma, tumor necrosis factor alpha.
Introduction

Fas antigen (CD95, Apo-1) is a 45-kilodalton transmembrane, cysteine-rich glycoprotein that belongs to the tumor necrosis factor receptor/nerve growth factor receptor superfamily (1, 2). Binding of Fas ligand to Fas antigen induces programmed cell death or apoptosis of susceptible cells (3-5). Recent studies have suggested that Fas ligand expressed on cytotoxic subsets of T lymphocytes may be one mechanism for T cell-mediated killing of cells expressing Fas antigen (6). The population of T cells expressing Fas ligand includes CD4+ as well as CD8+ subsets.

In sialoadenitis associated with Sjögren’s syndrome (SS), infiltrating cells are predominantly CD4 positive T lymphocytes (7). However, the mechanism of ductal destruction by infiltration of CD4+ lymphocytes remains unknown. We previously reported that de novo Fas antigen expression was observed on ductal epithelial cells in sialoadenitis with massive mononuclear cell infiltration in patients with SS (8), and some of the infiltrating mononuclear cells showed the Fas ligand (9). Apoptotic changes were observed in ductal epithelial cells and some infiltrating cells by DNA nick end labeling methods (9). These findings suggest that the Fas/Fas ligand system may play a role in the pathogenesis of sialoadenitis in SS by providing a specific target for cytotoxic T cells expressing the Fas ligand. Crosslinking of Fas antigen on ductal epithelium to Fas ligand on infiltrating lymphocytes may thereby induce apoptosis in ductal epithelial cells. However, the mechanism by which aberrant Fas antigen is expressed on ductal cells remains unknown.

A human salivary gland duct cell line (HSG) was established from irradiated human submandibular salivary gland. It has characteristics of intercalated duct cells (10). HSG cells are also able to mimic several features of the in vivo SS disease process (11). Cytokines such as IFN-γ and TNF-α induce the increased expression of ICAM-1 and HLA-DR in the HSG cell line.

In this study, we investigated the induction of Fas antigen and apoptosis by cytokines (IFN-γ, TNF-α, IL1-β, IL-2, IL-4, and GM-CSF) in the HSG cell line. IFN-γ and TNF-α increase Fas antigen expression and induce apoptotic cell death with anti-Fas antibody on HSG cells.

Materials and methods

Tissue culture

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 (Biowhittaker, MD, USA) at 37°C with 5% CO2.

Treatment of cells with cytokines

Recombinant human IFN-γ (R &D Systems, MN, USA), recombinant human TNF-α (Phamingen, CA, USA), recombinant human IL1-B (R &D Systems, MN, USA), human IL-2 (Boehringer Mannheim Biochemica), recombinant human IL-4 (Genzyme, MA, USA) and recombinant human GM-CSF (Genzyme, MA, USA) were used. Cells were cultured in the presence of IFN-γ 1-100 U/ml, TNF-α 1-100 U/ml, IL1-β 0.001-0.1 U/ml, IL-2 0.001-0.1 U/ml, IL-4 3-30 U/ml, or GM-CSF 0.01-0.1 ng/ml for 24 hours. To examine the time course of Fas expression on HSG, the cells were cultured in the presence of IFN-γ 100 U/ml or TNF-α 100 U/ml for 4 to 48 hours.

Cell ELISA

8 x 10^3 cells/well were seeded onto gelatin-coated 96-well flat-bottomed microtiter plates (Falcon 3072, Becton Dickinson, 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 incubated for various periods of time at 37°C in 5% CO2. The cultures were fixed with 3% paraformaldehyde in phosphate buffered saline (PBS) with 8% sucrose for 30 min at room temperature [subsequent washing steps were performed with 5 changes of PBS with 0.05% Tween 20® (Wako, Osaka, Japan)]. To avoid non-specific 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 0.1 ml of mouse anti-human Fas antibody (1 μg/ml) (DX-2) (Pharmingen, CA, USA) for 120 min at 37°C. After washing, the plates were treated with 0.1 ml/well of Envision solution® (peroxidase binding dextran polymer labeled with goat anti-mouse immunoglobulins) (Dako Japan, Kyoto, Japan) for 75 min at room temperature. After washing, the plates were incubated with 0.1 ml of 3,3',5,5' tetramethyl benzidine (Dako Japan, Kyoto, Japan) for 10 to 30 min at room temperature. The plates were read on a microtiter plate reader (SLT Lab Instruments, Salzburg, Austria) at 650 nm. For 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 wells used as negative controls. As the positive control for the cell ELISA system, epithelial membrane antibody (EMA Dako Japan, Kyoto, Japan) was used. The following negative controls were used: (a) omission of primary antibody or (b) appropriately diluted mouse IgG as a first layer.

Mouse immunoglobulin G blocking experiments
HSG cells were planted as described above and stimulated with 100 U/ml of IFN-γ for 24 hours. After treatment with anti-Fas antibody, Envision solution with 1 x 10^3 to 1 x 10^5 mg mouse immunoglobulin G was added as the second antibody.

Anti-IFN-γ blocking experiments
HSG cells were planted as described above and stimulated with 100 U/ml of IFN-γ and 0.2 to 5 μg/ml anti-IFN-γ antibody (Genzyme, MA, USA). Diluted IFN-γ was pretreated with anti-IFN-γ antibody for 30 min at 37°C. Fas antigen expression of HSG cells was assayed as described above.

Immunoperoxidase staining of cultured HSG on glass slides
1.6 x 10^4 cells/well were seeded onto Lab-Tek II ® chamber slides (Nunc, IL, USA) in 1 ml of medium. After reaching confluence (~48 hr), the culture medium was replaced with 1 ml of fresh medium with and without IFN-γ and incubated for 24 hours at 37°C in 5% CO₂. The preparation and staining of tissue sections for immunoperoxidase was carried out as follows: acetone fixed for 15 min at 4°C, and rinsed with phosphate buffered saline (PBS). After blocking by normal goat serum, the sections were incubated with the following solutions, each followed by PBS washing: 10 μg/ml of monoclonal antibody to Fas antigen (DX-2), Envision solution® (peroxidase binding dextran polymer labeled with goat anti-mouse immunoglobulins) (Dako Japan, Kyoto, Japan), 0.02% 3,3'-diaminobenzidine (DAB) containing 0.03% H₂O₂ and 10 mM sodium azide. The sections were subsequently stained with methyl green.

Immunofluorescence analysis
8 x 10^3 cells/well were seeded onto 96-well flat-bottomed microtiter plates (Falcon 3072, Becton Dickinson, 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 1 to 100 U/ml of IFN-γ or medium only and incubated for 24 hours at 37°C in 5% CO₂. These cells were incubated with 0.25 μg of DX-2 or 0.25 μg of mouse IgG for 30 min, the cells were washed 3 times in PBS and treated with FITC labeled goat anti-mouse IgG antibody (Becton Dickinson, CA, USA) for 30 min. The cells were analyzed using an FACScalibur (Becton Dickinson, CA, USA).

Detection of apoptotic cells by DNA nick end labeling
1.6 x 10^4 cells/well were seeded onto Lab-Tek II ® chamber slides (Nunc, IL, USA) in 1 ml of medium. After reaching confluence (~48 hr), the culture medium was replaced with 1 ml of fresh medium containing IFN-γ or TNF-α in various concentrations and incubated for 24 hours at 37°C in 5% CO₂. The chamber slides were incubated with or without 0.5 μg of anti-Fas antibody (DX-2) for 3 hours at 37°C. The cultures were fixed with ethanol for 30 min at room temperature. The appearance of DNA strand breaks during apoptosis was detected in the tissue by DNA nick end labeling methods (12). A multi-stage process of DNA fragmentation is typically associated with morphologic apoptosis. In several well-researched model systems, large fragments of 300 kb and 50 kb are first produced by endonucleolytic degradation of higher-order chromatin structural organization (13). The nuclei and apoptotic bodies in which new 3'-OH DNA ends were generated by DNA fragmentation were detected with an Apop Tag® in situ apoptosis detection kit (Oncor, Gaithersburg, MD). Normal or proliferative nuclei with low numbers of DNA 3'-OH do not stain with the kit. Residues of digoxigenin-nucleotide are catalytically added to the DNA by terminal deoxynucleotidyl transferase (TdT), an enzyme catalyzing a template-independent addition of deoxyribonucleotide triphosphate to the 3'-OH ends of double- or single-stranded DNA. The incorporated nucleotides form a random heteropolymer of digoxigenin-11-dUTP and dATP in a ratio that has been optimized for anti-digoxigenin antibody binding. The anti-digoxigenin antibody fragment carries a conjugated peroxidase to the reaction site. The number of apoptotic cells positively stained by DNA nick end labeling methods was recorded along with the total number of cultured cells. The percentage of stained apoptotic cells was then calculated. More than 100 HSG cells of three randomly selected areas on Lab-Tek ll® chamber slides containing each cytokine concentration were calculated for analysis.

Statistical analysis
Data analyses were performed using Student’s t-test in cell ELISA. In the analysis of apoptotic cells, we used Mann-Whitney’s U test.

Results
Expression of Fas antigen in the HSG cell line
By immunohistochemical staining on glass slides, Fas antigen was expressed on several cultured HSG cells without stimulation and this antigen expression was observed on many HSG cells stimulated by 100 U/ml of IFN-γ (Fig. 1). By immunofluorescence study, Fas antigen was mildly expressed on HSG cells and...
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Fig. 1. Immunohistochemical staining of anti-Fas antibody on cultured HSG cell line: (a) without cytokine stimulation, several HSG cells showed Fas antigen; (b) Fas antigen expression was observed in many HSG cells stimulated by 100 u/ml of IFN-γ for 24 hours.

Fig. 2. FACS study of Fas antigen expression on HSG cells. Fas antigen was mildly expressed on HSG cells without IFN-γ stimulation and this expression was upregulated by 100 u/ml of IFN-γ.

Fig. 3. Effect of increasing concentrations of anti-Fas antibody (DX-2) as first antibody on HSG and A673. Fas expression as measured by immunoperoxidase cell-ELISA. In the human Fas negative cell line L929, expression of Fas was not observed. All values are expressed as means ± SD of triplicate wells. No error bar indicates that the error was less than the symbol size.

this expression was upregulated by 100 u/ml of IFN-γ (Fig. 2). However, those analyses were not quantitative and were difficult to analyse quantitatively under many conditions.

Using an immunoperoxidase cell ELISA, we found that unstimulated HSG cells constitutively expressed low levels of Fas (Fig. 3). The expression of Fas was also measured by this cell ELISA in the Fas positive neuroepithelioma cell line A673 (24) and Fas expression was not observed in the human Fas negative mouse fibroblast cell line L929. This expression was essentially constant throughout all of the experiments. IFN-γ (1 - 100 U/ml) consistently upregulated constitutively expressed levels of Fas in a dose-dependent fashion (Fig. 4a). The time course of IFN-γ-induced Fas expression is shown in Figure 4b. A significant increase in Fas expression was observed 12 hours after the addition of IFN-γ (100 U/ml); Fas expression reached a plateau by 24 hours and remained stable up to 48 hours.

We further characterized this assay to determine if there were complications due to non-specific binding of the first or second antibody. For the negative control, appropriately diluted mouse IgG was used as first antibody. In that assay, optical density was less than 0.1. For the blocking assay for the second antibody, Envision solution with diluted mouse immunoglobulin G was added for second antibody, after blocking and treatment with anti-Fas antibody (Fig 4c). Addition of more than 1 x 10⁻³ mg of mouse IgG completely inhibited the activity of the second antibody.

To examine the specificity of IFN-γ, we tested the ability of antibody to IFN-γ to block the expression of Fas antigen. Pretreatment of IFN-γ with anti-IFN-γ antibody for 30 min before stimulation with IFN-γ resulted in dose-dependent elimination of the previously shown upregulative effect of Fas expression (Fig. 4d). 1 to 10 U/ml of TNF-α also upregulated constitutively expressed levels of Fas; these levels reached a plateau at 10 U/ml (Fig. 5a). The time course of TNF-α-induced Fas expression is shown in Figure 5b. A significant increase in Fas expression was observed 6 hours after the addition of TNF-α (100 U/ml); Fas ex-
Hypothalamus-pituitary-adrenocortical and -gonadal axis in RA / M. Cutolo

**Fig. 4.** Effect of IFN-γ on HSG Fas expression as measured by immunoperoxidase cell ELISA. Cells were cultured and stimulated with IFN-γ. (a) Fas expression after 24 hrs of exposure to increasing concentrations of IFN-γ. (b) Time course of the induction of Fas by IFN-γ (100 U/ml). (c) Effect of blockade by mouse immunoglobulin G on the second antibody. Addition of 10⁻³ mg or more of IgG to each well completely inhibited the activity of the second antibody. (d) Effect of blockade by anti-IFN-γ antibody. HSG cells were cultured and stimulated with 100 U/ml of IFN-γ and anti-IFN-γ antibody. Addition of 1 µg of IFN-γ inhibited the upregulation of IFN-γ. All values are expressed as means ± SD of triplicate wells. (*) indicates that the value was significantly different (*p < 0.01) from the control (untreated) OD. No error bar indicates that the error was less than the symbol size.

**Fig. 5.** Effect of TNF-α stimulation on HSG Fas expression as measured by immunoperoxidase cell ELISA. Cells were cultured and stimulated with TNF-α. (a) Fas expression after 24 hrs of exposure to increasing concentrations of TNF-α. (b) Time course of induction of Fas by TNF-α (100 U/ml). All values are expressed as means ± SD of triplicate wells. (*) indicates that the value was significantly different (*p < 0.01) from the control (untreated) OD.

Expression then reached a plateau and remained stable for up to 48 hours. 24 hours of incubation with IL-1β, IL-2, IL-4, or GM-CSF did not influence the expression of Fas antigen on HSG cells (Fig. 6 a-d).

**Fas-induced apoptosis in HSG cell line after stimulation with IFN-γ or TNF-α.** Apoptotic cell death was detected by DNA nick end labeling methods. Spontaneous apoptosis was observed in 4 to 6% of HSG cells after reaching confluence. After the addition of anti-Fas antibody, treatment with IFN-γ or TNF-α increased the percentage of apoptotic cells in a dose-dependent fashion. With more than 10 U/ml of IFN-γ or more than 1 U/ml of TNF-α, the percentages of apoptotic cells were statistically higher than those of unstimulated HSG cells (Fig. 7 a,b). The percentage of apoptotic cells stimulated by IFN-γ was 29 ± 3.6% and the percentage of TNF-α induced apoptotic cells was 21.3 ± 3.2%.

**Discussion**

Our study shows that HSG cells, human salivary intercalated duct cells transformed by irradiation, express Fas antigen after IFN-γ or TNF-α treatment and undergo apoptotic cell death in response to anti-Fas antibody. These results can hopefully serve as the basis for cellular models for future investigations addressing the regulation of Fas/Fas ligand expression and apoptosis in SS. Furthermore, we developed a modified cell ELISA system that appears to offer many advantages over conventional methods in quantifying membrane-bound Fas antigen.

Cytotoxic T lymphocytes (CTLs) can both specifically recognize and lyse their target. A well-known perforin-granzyme-based mechanism does not account for all examples of CTL lysis. Recently, the antigen-specific cytotoxicity expressed by *in vitro* and *in vivo* cytotoxic T cells was shown to be Fas based (14, 15). We previously reported that in sialoadenitis associated with SS *de novo* expression of Fas antigen was observed on the epithelial cells of the minor salivary ducts, especially in patients with severe mononuclear cell infiltration (8). Several infiltrating cells showed Fas ligand around
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Fig. 6. Effect of cytokines on HSG Fas expression. HSG cells were cultured and stimulated with IL-1β, IL-2, IL-4 and GM-CSF for 24 hrs. All values are expressed as means ± SD of triplicate wells. No significant increase in Fas expression was observed.

Fig. 7. Effect of increasing concentrations of IFN-γ and TNF-α on apoptosis of HSG cells induced by the addition of anti-Fas antibody. Cells were cultured and stimulated with IFN-γ and TNF-α. After culture with 0.5 µg of anti-Fas antibody for 3 hrs at 37°C, apoptotic cells were detected by the DNA nick end labeling method. Data represent the means ± SEM of three areas. (*) indicates the value was significantly different (p < 0.05) from the control (without addition of anti-Fas antibody) OD (Mann-Whitney’s U test).

the minor salivary ducts (9). Staining with DNA nick end labeling methods showed that ductal epithelial cells die by apoptosis (9). These findings suggest that the crosslinking of de novo Fas antigen on ductal epithelium to the Fas ligand on infiltrating lymphocytes induces the apoptosis of ductal epithelial cells. The mechanism of this aberrant Fas expression on ductal epithelial cells remains unknown.

The HSG cell line was established from submandibular salivary gland exposed to irradiation and has the characteristics of intercalated duct cells (10). Recently Wu et al. reported that apoptotic cell death was observed on HSG cells after 6 days of culture with IFN-γ ± TNF-α (11). HSG cells treated with 1000 U/ml of IFN-γ ± 20 U/ml of TNF-α did not display a true logarithmic growth phase within 5 days, and died with apoptotic changes after 6 days. They advocated that salivary cell death induced by IFN-γ and TNF-α could provide a source of extracellular nuclear antigen (i.e., SS-A, SS-B, and ANF) subsequently presented to T cells and could drive an immune response against autoantigen, further promoting the inflammatory process (16). They did not investigate the specific mechanisms by which cell death is induced in cytokine-treated HSG cells.

Our results show that IFN-γ or TNF-α induces Fas expression on salivary ductal cells and that Fas positive ductal cells
induced by those cytokines are susceptible to apoptosis by anti-Fas antibody. IFN-γ and TNF-α were shown by reverse transcriptase and a quantitative PCR methods to be produced by infiltrating lymphocytes and salivary epithelial cells (17, 18). IFN-γ and TNF-α secreted by infiltrating lymphocytes and salivary epithelial cells may induce Fas expression by, and apoptosis of, ductal cells. Fox et al. reported that IL-1β, IL-2, IL-4, IL-6, and IL-10 were secreted in salivary gland. In contrast, IL-1β, IL-2, IL-4, and GM-CSF did not influence on the levels of Fas in salivary ductal cells. TNF-α also upregulated constitutively expressed levels of Fas. However, levels of Fas antigen stimulated by TNF-α on HSG cells were lower than those stimulated by IFN-γ. TNF-α, by itself, seems to have less effect than IFN-γ on HSG cells. TNF-α is associated with significantly increased secretion of IL-6 by HSG cells (19). Wu et al. reported that TNF-α can upregulate the number of IFN-γ receptors on HSG cells (20, 21). TNF-α may amplify the effects of IFN-γ and other cytokines. TNF-α-induced Fas expression reached a plateau by 6 hours and remained stable for up to 48 hours, whereas IFN-γ-induced Fas expression reached a plateau by 24 hours and remained stable up to 48 hours. HSG cells stimulated with TNF-α have an earlier plateau of Fas expression than do cells stimulated with IFN-γ. The reasons for the difference in the time to plateau levels are unknown. IFN-γ-induced Fas expression might require some other stimuli (i.e. cytokines) produced by HSG cells stimulated by IFN-γ.

A variety of methods, including FACs and radioimmunoassay, have been used to measure Fas antigen expression. Cell ELISA was compared with immunofluorescence staining by Gronow et al. (22) and the two assays were found to be similarly sensitive. The CD4 positive cell line and CD4 negative cell line were mixed at different cell ratios. Anti-CD4 curves in the cell ELISA and mean immunofluorescence intensities both reached cut-off values at a cell ratio of 12.5%. Those results underline the application of the cell ELISA for antigen detection on homogeneous cell lines. We used 3% paraformaldehyde in PBS with 8% sucrose fixation for cell ELISA because microtiter plate became clouded by sucrose fixation. Gronow et al. reported that the cell ELISA gave similar results when fresh or 1% paraformaldehyde fixed cells were used. We also measured the Fas expression by cell ELISA without fixation. Fas expression on HSG cells could be assayed, but many HSG cells were observed to be detached from the plate (data not shown). HSG cells fixed by 3% paraformaldehyde in PBS with 8% sucrose were observed to be well adherent to the plate. Immuno-alkali phosphatase cell ELISA is often used to assay cell surface antigen (22). However, the endogenous alkali-phosphates activity of HSG cells is so strong that it cannot be blocked. Immunoperoxidase methods are often used to histochemically stain tissue sections or cultured cells (23), and have therefore been used to visualize Fas antigen.

We used this procedure to quantify the relative levels of Fas expression in the HSG cell line. The results obtained with this simple immunoperoxidase assay are comparable to those obtained with the procedures described above. In contrast to FACs and radioimmunoassay, our approach avoids mechanical disruption of cell monolayers, trypsinization, and the use of radioactivity (22, 23). Our findings suggest that HSG cells, in conjunction with this simple immunoperoxidase cell ELISA, provide a reliable system for studying modulation of Fas expression on salivary duct cells and ductal destruction in SS.

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References

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