

# Expression and Function of X Chromosome-Linked Inhibitor of Apoptosis Protein in Sjögren's Syndrome

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**SUMMARY:** Apoptotic cell death in acinar and ductal epithelial cells is thought to play an important role in the development of salivary gland dysfunction in patients with Sjögren's syndrome (SS). We examined the expression of anti-apoptotic molecules in salivary glands from patients with SS. The labial salivary glands from six human T-cell leukemia virus (HTLV)-I-seronegative and eleven HTLV-I-seropositive SS patients were analyzed by immunohistochemistry. In vitro experiments were performed with a human salivary gland cell line (HSG cells). Immunohistologic analyses revealed that Bcl-2 and Bcl-x were preferentially expressed in salivary infiltrating mononuclear cells more than acinar and ductal epithelial cells. In contrast, strong X chromosome-linked inhibitor of apoptosis protein (XIAP) expression was evident in both acinar and ductal epithelial cells. The pattern of expression of these anti-apoptotic molecules was similar in both HTLV-I-seropositive and HTLV-I-seronegative SS patients. Western blot analysis confirmed expression of XIAP in cultured HSG cells. The expression of XIAP in HSG cells was increased by IL-1 $\beta$ , TGF- $\beta$ 1, or IL-10. However, XIAP expression was down-regulated by TNF- $\alpha$ , which induced apoptotic cell death of HSG cells with an increase in caspase-3 activity. These effects of TNF- $\alpha$  in HSG cells were antagonized by IL-1 $\beta$ , TGF- $\beta$ 1, or IL-10. Our results suggest that XIAP is important in regulating apoptotic cell death of acinar and ductal epithelial cells in patients with SS. (*Lab Invest* 2000, 80:1421-1427).

Apoptosis, or programmed cell death, is an essential mechanism for the selective elimination of cells. Deficient or accelerated apoptotic cell death is associated with a variety of disorders, encompassing most cell systems (Thompson, 1995). Sjögren's syndrome (SS) is an organ-specific autoimmune disorder characterized by an infiltration of activated lymphocytes into the salivary glands and destruction of the glandular structure (Klippel and Dieppe, 1998; Koopman, 1997). Recent reports reveal that apoptotic cell death is preferentially found in acinar and ductal epithelial cells more than salivary infiltrating mononuclear cells (MNC) (Kong et al, 1997; Manganelli et al, 1997; Nakamura et al, 1998). This may be a mechanism mediating loss of secretory function of SS. However, the molecular interactions leading to apoptosis of acinar and ductal epithelial cells remain to be clarified.

Caspase-3 is a major effector molecule in inducing apoptotic cell death (Nagata, 1997). The activation of caspase-3 is positively or negatively regulated by at least two kinds of protein families, Bcl-2-related pro-

teins and inhibitor of apoptosis proteins (IAP). Activation of caspase-3 is inhibited by Bcl-2 or Bcl-xL and accelerated by Bax (Chao and Korsmeyer, 1998; Nagata, 1997; Reed, 1997). X chromosome-linked inhibitor of apoptosis protein (XIAP), which is a member of the IAP family of proteins, inhibits caspase-3 activation (Deveraux et al, 1997, 1998).

We previously reported a high prevalence of human T-cell leukemia virus (HTLV)-I Ab in SS patients from certain endemic areas in Japan (Eguchi et al, 1992; Terada et al, 1994). Additionally, a high prevalence of SS in patients with HTLV-I-associated myelopathy (HAM) has also been reported (Nakamura et al, 1997), suggesting that HTLV-I is one of the causative agents of SS. In the present study, we examined the expression of anti-apoptotic molecules expressed in acinar and ductal epithelial cells in salivary glands of SS patients with or without HTLV-I seropositivity. We also investigated the expression and functional properties of XIAP in cultured human salivary gland cell line (HSG cells).

## Results

### **Expression of Bcl-2-Related Proteins and XIAP in Labial Salivary Glands of SS Patients**

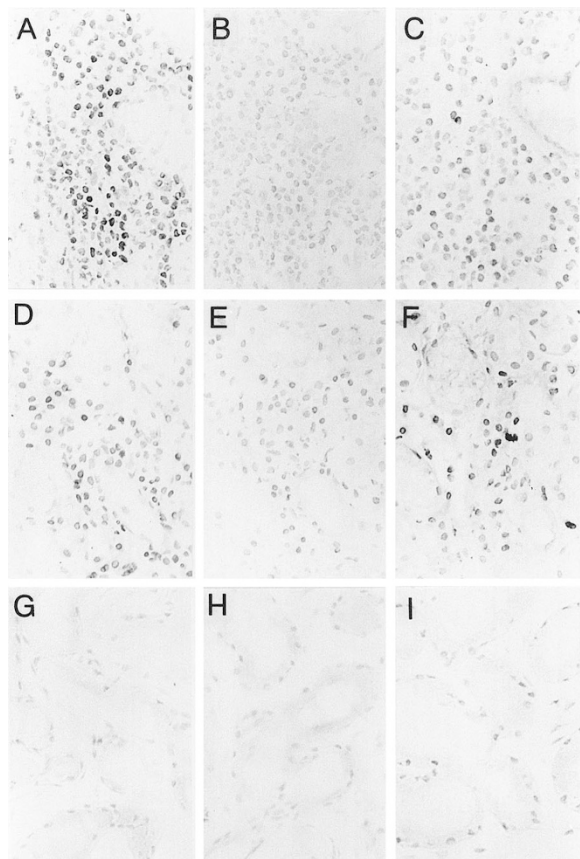
We initially examined the expression of Bcl-2-related proteins and XIAP in labial salivary glands of SS patients. As we recently described (Nakamura et al,

Received May 3, 2000.

This work was supported in part by a Grant-in-Aid (05670426) from the Ministry of Education, Science, Sport and Culture, Japan.

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1999), more than 50% of infiltrating MNC expressed both Bcl-2 and Bcl-x, irrespective of HTLV-I seropositivity. However, Bax was expressed in only a few infiltrating MNC (Fig. 1). The expression of these Bcl-2-related proteins in acinar and ductal epithelial cells was weak compared with the expression in salivary infiltrating MNC (Fig. 1). Expression of Bcl-2, Bcl-x, and Bax was not observed in sections from control subjects (Fig. 1). Although the expression of Bcl-2-related proteins was not obvious in either acinar or ductal epithelial cells, strong XIAP expression was determined in both cell types from SS patients, irrespective of HTLV-I seropositivity (Fig. 2). Expression of XIAP was not observed in sections from control subjects (Fig. 2). In contrast to acinar and ductal epithelial cells, XIAP expression was not clear in salivary infiltrating MNC (Fig. 2).



**Figure 1.**

Expression of Bcl-2, Bax, and Bcl-x in labial salivary glands of SS patients with or without HTLV-I antibody. *A to C*, Representative HTLV-I-seropositive SS patient. *D to F*, Representative HTLV-I-seronegative SS patient. *G to I*, Control subject. Note that the major population of salivary infiltrating MNC from SS patients express both Bcl-2 (*A and D*) and Bcl-x (*C and F*), whereas the percentage of Bax<sup>+</sup> MNC (*B and E*) was quite low when compared with that of Bcl-2<sup>+</sup> or Bcl-x<sup>+</sup> MNC. No differences were detected between HTLV-I-seropositive and HTLV-I-seronegative SS patients. Expression of these Bcl-2-related proteins in acinar/ductal epithelial cells was weak when compared with MNC expression. Expression of Bcl-2 (*G*), Bax (*H*), or Bcl-x (*I*) was not observed in the labial salivary glands of control subjects. Original magnification,  $\times 400$ .

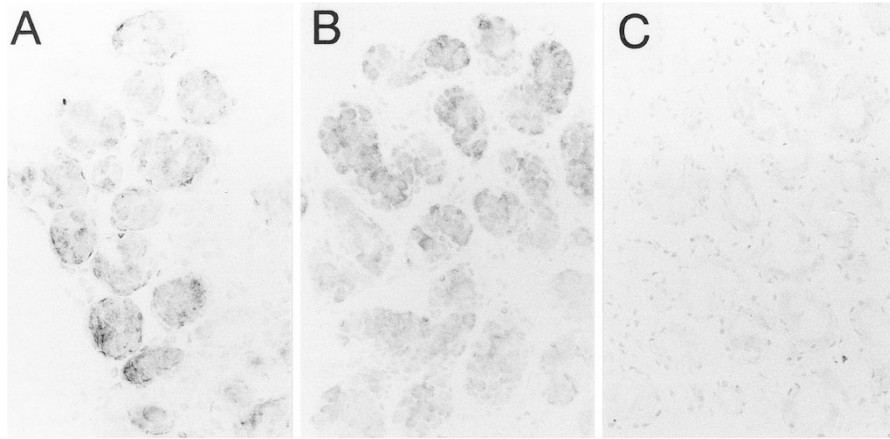
### Modulation of XIAP Expression in HSG Cells by Cytokines

Our immunohistochemistry results suggested that XIAP was an important molecule regulating apoptosis in acinar and ductal epithelial cells. Various cytokines have been detected in situ in salivary glands of SS patients (Klippel and Dieppe, 1998; Koopman, 1997). Therefore, we investigated whether cytokines could modulate the expression of XIAP, which regulates an apoptotic process, in cultured HSG cells. In concordance with previous reports (Klippel and Dieppe, 1998; Koopman, 1997), strong expression of IL-1 $\beta$ , TNF- $\alpha$ , transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1), and IL-10 was detected in salivary glands from SS patients (Fig. 3). TNF- $\alpha$  expression was mainly confined to infiltrating MNC. IL-1 $\beta$ , TGF- $\beta$ 1, and IL-10 were expressed in both infiltrating MNC and acinar/ductal epithelial cells (Fig. 3). Culture of HSG cells with TNF- $\alpha$  decreased XIAP expression (Fig. 4). A clear induction of apoptosis was found in HSG cells treated with TNF- $\alpha$ ; along with an increase in caspase-3 activity, XIAP expression was markedly decreased (Figs. 4, 5, and 6). The addition of Ac-DEVD (Asp-Glu-Val-Asp)-aldehyde almost completely suppressed the appearance of hypodiploid DNA<sup>+</sup> HSG cells induced by TNF- $\alpha$  (data not shown). This suggests that caspase-3 is a major effector molecule in the TNF- $\alpha$ -induced apoptosis of HSG cells. In contrast, XIAP expression in HSG cells was increased by IL-1 $\beta$ , TGF- $\beta$ 1, or IL-10 (Fig. 4). The decrease of XIAP expression by TNF- $\alpha$  in HSG cells was antagonized by the presence of IL-1 $\beta$ , TGF- $\beta$ 1, or IL-10, almost to the levels found in unstimulated HSG cells (Fig. 7). Induction of apoptotic cell death and caspase-3 activity in HSG cells by TNF- $\alpha$  was suppressed, although not completely inhibited, by IL-1 $\beta$ , TGF- $\beta$ 1, or IL-10 (Figs. 5 and 6). The in situ expression of IL-10 receptor (IL-10R) was confirmed in acinar and ductal epithelial cells of SS patients and in cultured HSG cells (Fig. 8).

### Discussion

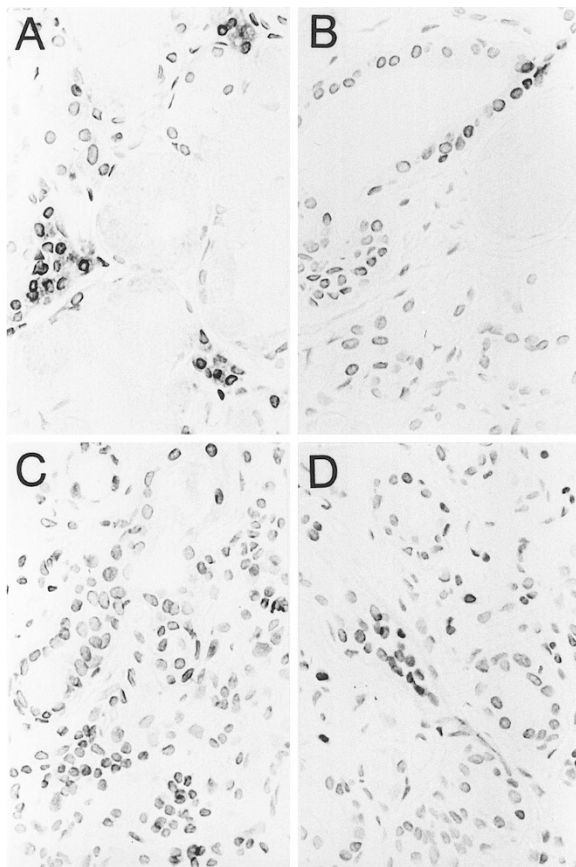
Recent immunohistologic examinations revealed the presence of apoptotic cell death in salivary glands from patients with SS, in acinar and ductal epithelial cells more than in salivary infiltrating MNC (Kong et al, 1997; Manganelli et al, 1997; Nakamura et al, 1998). Therefore, an apoptotic cell death of acinar and ductal epithelial cells could be a major mechanism perpetuating the destruction of the salivary gland.

Here, immunohistologic analysis suggested that XIAP is an important anti-apoptotic molecule expressed in acinar and ductal epithelial cells. The expression of Bcl-2 and Bcl-x was quite weak in acinar and ductal epithelial cells compared with XIAP expression. Therefore, we examined the expression and function of XIAP in cultured HSG cells. XIAP is a member of IAP family proteins, which inhibit caspase-3 activation (Deveraux et al, 1997, 1998). Cytokines or adhesion molecules could be extrinsic factors involved in the modulation of apoptosis. Various cytokines that might modulate the expression of



**Figure 2.**

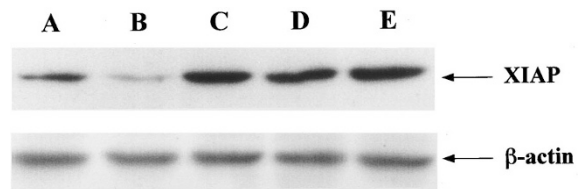
Expression of XIAP in acinar and ductal epithelial cells in labial salivary glands of SS patients. *A*, Representative HTLV-I-seropositive SS patient. *B*, Representative HTLV-I-seronegative patient. *C*, Control subject. Note the strong XIAP expression in both acinar and ductal epithelial cells from SS patients and the absence of XIAP expression in acinar and ductal epithelial cells of control subjects. In contrast to acinar/ductal epithelial cells, XIAP expression was not clear in MNC. No differences were observed between HTLV-I-seronegative and HTLV-I-seropositive SS patients. Original magnification,  $\times 200$ .



**Figure 3.**

Cytokines expressed in labial salivary glands from SS patients. Representative HTLV-I-seronegative SS patient. *A*, TNF- $\alpha$ ; *B*, IL-1 $\beta$ ; *C*, TGF- $\beta$ 1; *D*, IL-10. Note that TNF- $\alpha$  was expressed in salivary infiltrating MNC, but not in acinar or ductal epithelial cells. IL-1 $\beta$ , TGF- $\beta$ 1, and IL-10 were expressed in both infiltrating MNC and acinar/ductal epithelial cells. Original magnification,  $\times 400$ .

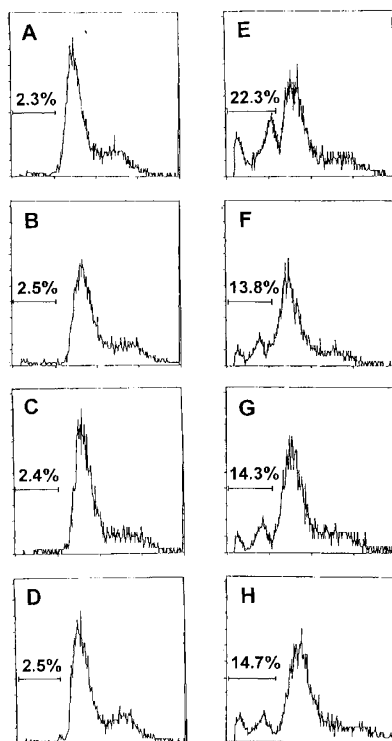
XIAP in acinar and ductal epithelial cells are produced in the salivary glands of SS. We examined the modulation of XIAP expression in cultured HSG cells. HSG



**Figure 4.**

XIAP expression in HSG cells determined by Western blotting. HSG cells were cultured with or without cytokines for 72 hours and XIAP expression was determined by Western blot analysis. *Lane A*, Untreated HSG cells. *Lanes B* to *E*, XIAP expression in HSG cells treated with TNF- $\alpha$  (*lane B*), IL-1 $\beta$  (*lane C*), TGF- $\beta$ 1 (*lane D*), and IL-10 (*lane E*). Note that XIAP expression in HSG cells was decreased by TNF- $\alpha$ , whereas XIAP expression was increased by IL-1 $\beta$ , TGF- $\beta$ 1, and IL-10. Results are representative of five experiments.

cells did not undergo spontaneous apoptosis in culture, and clearly expressed XIAP. Culture of HSG cells with IL-1 $\beta$ , TGF- $\beta$ 1, or IL-10 increased the expression of XIAP. In contrast, the expression of XIAP in HSG cells was significantly reduced by TNF- $\alpha$ , and apoptotic cell death with an increase in caspase-3 activity was found in the TNF- $\alpha$ -treated HSG cells. This indicates a functional significance of XIAP in regulating the apoptotic cell death of HSG cells. When HSG cells were cultured with TNF- $\alpha$  in the presence of IL-1 $\beta$ , TGF- $\beta$ 1, or IL-10, the TNF- $\alpha$ -induced apoptosis of HSG cells with increase of caspase-3 activity was inhibited by IL-1 $\beta$ , TGF- $\beta$ 1, or IL-10. With IL-1 $\beta$ , TGF- $\beta$ 1, or IL-10, the decrease of XIAP expression in TNF- $\alpha$ -treated HSG cells was almost recovered to levels found in unstimulated HSG cells. These results suggest that cytokines produced in the salivary glands in SS regulate apoptosis of acinar and ductal epithelial cells by modulating XIAP expression. However, anti-apoptotic molecules other than XIAP, such as cIAP1, cIAP2, or survivin (Deveraux et al 1998; Tamm et al, 1998), could be involved in TNF- $\alpha$ -induced HSG cell apoptosis because approximately half of the caspase-3 activity-inducing apoptosis of TNF- $\alpha$ -



**Figure 5.**

Detection of apoptosis in HSG cells by flow cytometry. HSG cells were cultured with or without cytokines for 72 hours and apoptotic cell death was determined by flow cytometric analysis. *A*, Untreated HSG cells. *B*, IL-1β-treated HSG cells. *C*, TGF-β1-treated HSG cells. *D*, IL-10-treated HSG cells. *E*, TNF-α-treated HSG cells. *F*, HSG cells treated with TNF-α and IL-1β. *G*, HSG cells treated with TNF-α and TGF-β1. *H*, HSG cells treated with TNF-α and IL-10. Note that the induction of apoptotic cell death in HSG cells by TNF-α was antagonized by IL-1β, TGF-β1, or IL-10. Results are representative of five experiments.

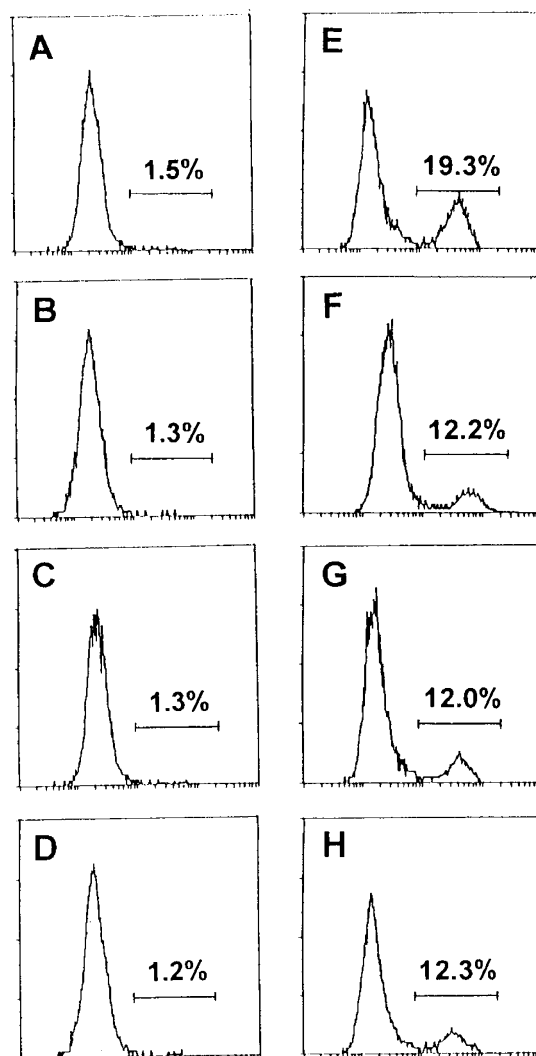
treated HSG cells still remained after the addition of IL-1β, TGF-β1, or IL-10.

We have demonstrated that apoptosis of acinar and ductal epithelial cells is modulated by XIAP, and that XIAP expression could be positively or negatively regulated by cytokines expressed in salivary glands of SS patients. We also found that XIAP expression was independent of HTLV-I-seropositivity, indicating that common molecular mechanisms may regulate apoptosis in the salivary glands, independent of the pathologic organism. Our findings may also provide new insight into the development of cell-type-specific immunotherapy for SS by modulating the expression of apoptosis-related molecules.

## Materials and Methods

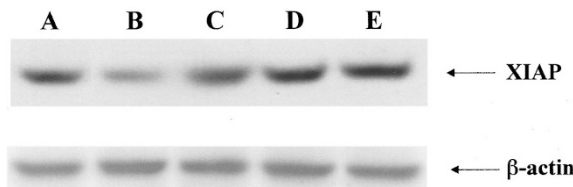
### Patients

All patients were female and fulfilled the criteria for diagnosis of SS as defined by the European Community (Vitali et al, 1993). Six HTLV-I-seronegative (48.2 ± 26.5 years old, range: 19 to 86 years old) and eleven HTLV-I-seropositive SS patients were entered in the study. The latter group comprised five patients with HAM (59.0 ± 11.7 years old, range: 40 to 72) and six patients without HAM (54.0 ± 15.1 years old,



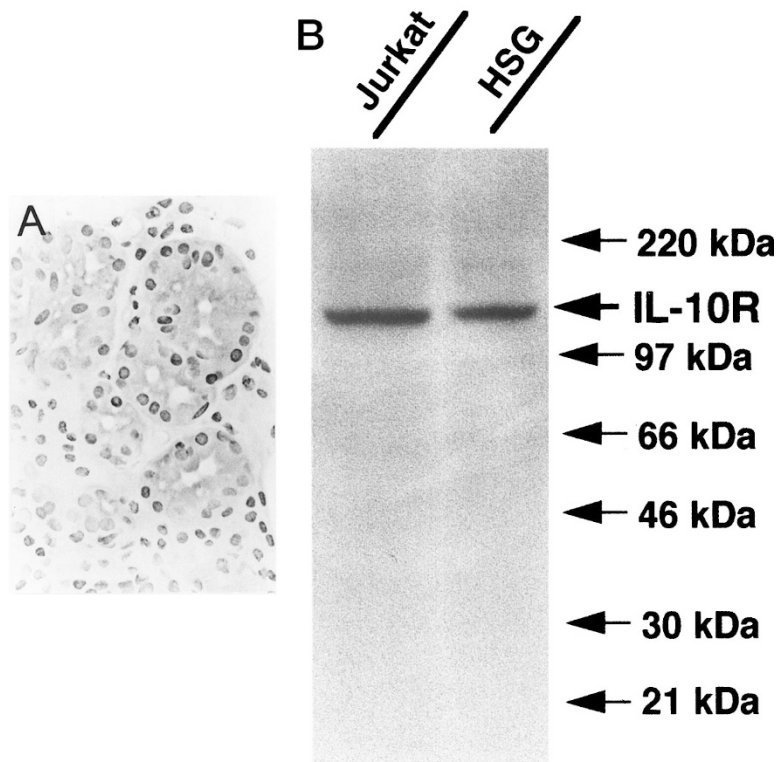
**Figure 6.**

Intracellular caspase-3 activity in HSG cells determined by flow cytometry. HSG cells were cultured with or without cytokines for 72 hours and intracellular caspase-3 activity was determined by flow cytometric analysis. *A*, Untreated HSG cells. *B*, IL-1β-treated HSG cells. *C*, TGF-β1-treated HSG cells. *D*, IL-10-treated HSG cells. *E*, TNF-α-treated HSG cells. *F*, HSG cells treated with TNF-α and IL-1β. *G*, HSG cells treated with TNF-α and TGF-β1. *H*, HSG cells treated with TNF-α and IL-10. Note that the TNF-α-induced intracellular caspase-3 activity in HSG cells by was antagonized by IL-1β, TGF-β1, or IL-10. Results are representative of five experiments.



**Figure 7.**

TNF-α-induced decrease in XIAP expression in HSG cells is antagonized by IL-1β, TGF-β1, or IL-10. HSG cells were cultured with or without cytokines for 72 hours and XIAP expression was determined by Western blot analysis. *Lane A*, Untreated HSG cells. *Lane B*, TNF-α-treated HSG cells. *Lane C*, HSG cells treated with TNF-α and IL-1β. *Lane D*, HSG cells treated with TNF-α and TGF-β1. *Lane E*, HSG cells treated with TNF-α and IL-10. Note that the TNF-α-induced decrease in XIAP expression in HSG cells was antagonized by IL-1β, TGF-β1, or IL-10. Results are representative of five experiments.



**Figure 8.**

IL-10R expression determined by immunohistochemistry and Western blotting. *A*, IL-10R expression in the labial salivary glands of SS patients determined by immunohistochemistry. Note that IL-10R was detected in both infiltrating MNC and acinar/ductal epithelial cells. Original magnification,  $\times 400$ . *B*, IL-10R expression in HSG cells determined by Western blotting. Jurkat cells were used as positive controls. IL-10R was clearly detected in cultured HSG cells.

range: 40 to 72). The five HAM patients were diagnosed according to the criteria proposed by Osame et al (1987). Control subjects had sicca symptoms, but were not diagnosed as SS. Patients with SS secondary to other autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, or progressive systemic sclerosis, were not included. Informed consent was obtained from all participating subjects and the study was conducted in accordance with the human experimental guidelines of our institution.

#### ***Biopsy of Labial Salivary Glands***

Biopsies of minor labial salivary glands were obtained from the mucosa of the lower lip, 0.5 to 1.0 cm lateral to midline, under local anesthesia. Tissues were fixed in 4% paraformaldehyde in PBS (pH 7.4) immediately after biopsy, and were successively immersed in 10%, 15%, and 20% sucrose before being frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until use.

#### ***Monoclonal and Polyclonal Abs for Immunohistochemistry***

A polyclonal antibody that recognizes the N-terminal portion of Bax and a monoclonal antibody to TGF- $\beta 1$  were purchased from Santa Cruz Biotechnology (Santa Cruz, California). A monoclonal antibody to Bcl-2 was purchased from Dako (Glostrup, Denmark). A polyclonal antibody to Bcl-x, which recognizes both

the Bcl-xS and the Bcl-xL isoforms, was purchased from Transduction Laboratories (Lexington, Kentucky). A monoclonal antibody to XIAP was purchased from MBL (Nagoya, Japan). IL-10 monoclonal and IL-10R polyclonal antibodies were purchased from R&D Systems (Minneapolis, Minnesota). Monoclonal antibodies to TNF- $\alpha$  and IL-1 $\beta$  were purchased from Genzyme (Cambridge, Massachusetts).

#### ***Immunohistochemical Examination of Salivary Glands***

Four-micron thick tissue sections of labial salivary glands were cut and mounted on aminopropyltriethoxysilane-coated glass slides. The streptavidin-biotin method (Histofine Staining Kit; Nichirei Company, Tokyo, Japan) was used for immunohistochemical detection, as described previously (Nakamura et al, 1997). Briefly, endogenous peroxidase was inactivated by immersing the section in a 3% hydrogen peroxide solution. Sections were incubated with 10% goat or rabbit serum, then incubated with primary antibody in a humidified chamber for 60 minutes at room temperature (except the IL-10R antibody, which was incubated overnight). The sections were next incubated with the appropriate biotinylated anti-rabbit or anti-mouse IgG for 12 minutes, washed, and incubated with peroxidase-conjugated streptavidin. Color was developed using 3,3'-diaminobenzidine and hydrogen peroxide. Negative control sections were incubated with either normal mouse IgG or normal rabbit serum.

### Effect of Cytokines on Apoptotic Cell Death in Cultured HSG Cells

The HSG cell line (Sato et al, 1985), established from a human salivary gland, was a kind gift from Dr. Yoshio Hayashi (Tokushima University School of Dentistry, Tokushima, Japan). HSG cells ( $1 \times 10^5$  cells per well in 6-well tissue culture plates, Costar 3516; Costar, Cambridge, Massachusetts) were cultured in E-MEM containing 2% BSA in the presence or absence of TNF- $\alpha$  (200 IU/ml; Kirin, Tokyo, Japan), IL-1 $\beta$  (20 IU/ml; Otsuka Pharmaceutical Company, Tokushima, Japan), IL-10 (20 ng/ml, Genzyme), or TGF- $\beta$ 1 (5 ng/ml, Kirin) for 72 hours. After incubation, apoptotic cell death, activation of caspase-3, and the expression of XIAP in HSG cells were examined. Apoptotic cell death was quantified by the percentage of cells with hypodiploid DNA as previously described (Kawakami et al, 1999). Briefly, treated HSG cells were fixed with 70% ethanol, treated with RNase (100  $\mu$ g/ml, Sigma Chemical Company, St. Louis, Missouri), and stained with propidium iodide (100  $\mu$ g/ml, Sigma) for 30 minutes, on ice. The stained cells were analyzed by a flow cytometer (Epics XL, Beckman Coulter, Hialeah, Florida) to detect the presence of the cells with hypodiploid DNA.

We examined intracellular caspase-3 activity in HSG cells by flow cytometry using the PhiPhiLux G1D2 Kit (MBL). Briefly, treated HSG cells were centrifuged to remove the culture medium. DEVD substrate containing rhodamine was added to the cell pellet and incubated in a 5% CO<sub>2</sub> incubator at 37° C for 60 minutes. After incubation, samples were analyzed by flow cytometer (Epics XL) to determine the percentage of intracellular active caspase-3<sup>+</sup> cells. To examine the functional significance of caspase-3 in apoptotic process of HSG cells, 300  $\mu$ M of Ac-DEVD-aldehyde (DEVD-CHO, Peptide institute, Osaka, Japan) was added to HSG cells 3 hours before the addition of cytokines. The percentage of cells with hypodiploid DNA was determined as described above.

### Western Blot Analysis for the Expression of XIAP and IL-10R in HSG Cells

HSG cells were collected after incubation in the presence or absence of TNF- $\alpha$  (200 IU/ml, Kirin), IL-1 $\beta$  (20 IU/ml, Otsuka Pharmaceutical Company), IL-10 (20 ng/ml, Genzyme), or TGF- $\beta$ 1 (5 ng/ml, Kirin) for 72 hours in E-MEM supplemented with 2% BSA as described above, and lysed with lysis buffer (1% NP-40; 50 mM Tris, pH 7.5; 100 mM NaCl; 5 mM EDTA; 1 mM polymethylsulfonfylfluoride) for 20 minutes at 4° C. Insoluble materials were removed by centrifugation at 14,000 rpm for 30 minutes at 4° C. Supernatants were collected, and protein concentrations determined using the Bio-Rad protein assay kit (Melville, New York). An identical amount of protein (20  $\mu$ g) for each lysate was subjected to 10% SDS-PAGE, before being transferred to nitrocellulose filters. Filters were blocked for 1.5 hours using 5% nonfat dried milk in TBS (50 mM Tris, 0.15 M NaCl, pH 7.5) containing

0.1% Tween 20, washed with TBS, and incubated at room temperature for 2 hours in a 1:1000 dilution of XIAP mAb or IL-10R Ab. Filters were washed with TBS and incubated with a 1:1000 dilution of secondary antibody coupled to horseradish peroxidase. The enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, Illinois) was used for detection. A monoclonal antibody to  $\beta$ -actin (Sigma) was used as an internal control protein.

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