Thioredoxin May Exert a Protective Effect Against Tissue Damage Caused by Oxidative Stress in Salivary Glands of Patients with Sjögren's Syndrome

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ABSTRACT. ***Objective.*** To demonstrate the existence of oxidative stress and the role of the antioxidant thioredoxin (TRX) in Sjögren's syndrome (SS).

Methods. Labial biopsy specimens from patients with SS were analyzed immunohistochemically to detect 8-hydroxy-2′-deoxyguanosine (8-OHdG), 4-hydroxy-2-nonenal (4-HNE), nitrotyrosine, and TRX. Levels of TRX in saliva and plasma were quantified by ELISA. To analyze the effect of TRX on human salivary gland (HSG) cells, recombinant TRX (rTRX)-treated HSG cells were stimulated by interferon-γ (IFN-γ) for detecting interleukin 6 (IL-6) with ELISA and RT-PCR, or stimulated with IFN-γ and anti-Fas antibody for analyzing Fas-induced apoptosis with PI/annexin V staining.

Results. Large amounts of 8-OHdG, 4-HNE, nitrotyrosine, and TRX were produced in salivary duct cells of SS patients, whether there was periductal lymphocytic infiltration or not. Strong TRX expression was detected in acinar cells from 13 of 19 SS specimens. Levels of salivary TRX were significantly higher in SS patients than in controls ($p < 0.05$), and were inversely related to the salivary flow rates in SS patients. Patients who showed acinar TRX expression had higher salivary TRX levels than those who did not ($p < 0.05$). Interferon-γ-induced expression of IL-6 and Fas-mediated apoptosis in HSG cells were significantly suppressed by pretreating cells with rTRX.

Conclusion. Parallel production of oxidative stress markers together with massive secretion of TRX suggests that oxidative stress induces TRX in the salivary gland. Moreover, suppression of IL-6 production and apoptosis by rTRX in HSG cells suggests TRX acts to protect the salivary glands of SS patients from tissue damage. (First Release Sept 15 2007; J Rheumatol 2007;34:2035–43)

Key Indexing Terms:
SJÖGREN'S SYNDROME THIOREDOXIN 8-OHdG OXIDATIVE STRESS DUCTAL CELL

Sjögren's syndrome (SS) is a chronic inflammatory disorder characterized by lymphocytic infiltration of the lacrimal and salivary glands. While the key histological feature of SS is focal accumulation of lymphocytes, salivary epithelial cells are also suggested to play a role in the disease pathogenesis[1-3]. In SS, glandular epithelial cells inappropriately express high levels of class II MHC and possibly present antigens to invading T cells[4]. Cytokine production including interleukin 2 (IL-2), IL-6, IL-10, tumor necrosis factor-α (TNF-α), transforming growth factor-ß, and interferon-γ (IFN-γ) from salivary epithelial cells has been observed using the cell-specific microdissection technique[5].

We and others have reported that oxidative stresses such as ultraviolet-B and a chemical oxidant, diamide, induced cell-surface expression of SSA/Ro-52 antigen[6,7], which might be the process responsible for antigen-presenting of SSA/Ro-52 to T cells in SS and the other SSA-positive autoimmune diseases. Thus, oxidative stress might be involved in the immune process at the early stage of SS pathogenesis. Reactive oxygen species (ROS) have been attracting attention as causative agents in the pathogenesis of human diseases including autoimmune disorders[8]. For instance, lymphocytes isolated from patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) contain high levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG)[9-11], which is an established marker of DNA damage caused by oxidative stress[12]. Urinary

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excretion of 8-OHdG is thought to be a good indicator of oxidative stress[13]. The damage caused by ROS alters the structure of DNA and its immunogenicity, perhaps resulting in generation of autoantibodies that are cross-reactive with DNA[10,14]. We reported that both urinary and plasma 8-OHdG levels were significantly elevated in patients with SS[8]. Determination of 8-OHdG levels within specific tissues should improve our understanding of the role played by oxidative stress in the development and progression of autoimmune diseases.

Thioredoxin (TRX) is a ubiquitous antioxidant protein[15,16]. Once expressed, TRX functions as an important scavenger of ROS[17]. In addition, TRX is a key regulator of thiol oxidation and apoptosis signal-regulating kinase 1, which is activated by ROS[18], and it also has multifunctional immunological properties, including cytokine-like activity and ability to stimulate lymphocyte activation[19-21]. Thus TRX expression in tissues affected by autoimmune diseases suggests it does not function simply as an antioxidant responding to oxidative stress; it also acts as an immunomodulator.

We hypothesized that oxidative stress might play an important role in SS pathogenesis and that the antioxidant TRX might act as an immunomodulator as well as an antioxidant in the process. To prove this, we evaluated the distribution of oxidative stress markers and TRX in salivary gland tissues, titrated salivary TRX levels from SS patients, and analyzed the role of TRX *in vitro* by utilizing the salivary gland tumor-derived cell line, HSG.

MATERIALS AND METHODS

Patients and controls. Twenty-nine female patients with SS (mean age 51 ± SD 14.3 yrs) at Kobe University Hospital were enrolled in the study. All were diagnosed as having SS based on the revised Japanese criteria for primary or secondary SS (1999; Table 1). Control groups comprised 13 RA patients (age 53.5 ± 15.6 yrs) and 9 SLE patients (36.6 ± 9.1 yrs) without sicca symptoms, and 19 healthy donors (48.6 ± 11.4 yrs) for saliva examinations. For histological examinations, 2 healthy donors and 3 patients with nonspecific sialadenitis with sicca symptoms were enrolled.

This work was carried out in compliance with the Helsinki Declaration, with the approval of the Ethics Committee for Human Research at Kobe University Medical School. With informed consent, samples were obtained from patients who underwent labial biopsies for diagnosis of SS. Specimens were obtained from 19 SS patients and 2 control subjects.

Immunohistochemical analysis for labial tissues. Paraffin sections were deparaffinized, dehydrated, and autoclaved for unmasking of antigen for 5 min. After elimination of endogenous peroxidase activity by incubation for 30 min with 0.3% H_2O_2, the sections were incubated overnight at 4°C with mouse anti-human TRX (15 µg/ml, monoclonal), anti-8-OHdG (10 µg/ml; Oxis International, Portland, OR, USA), anti-4-HNE (25 µg/ml; Japan Institute for the Control of Aging, Shizuoka, Japan), antinitrotyrosine (10 µg/ml; Sigma, St. Louis, MO, USA), and anti-human HLA-DR (Nichirei, Tokyo, Japan) antibodies. Normal mouse IgG or rabbit IgG was used as a negative control. Sections were washed with phosphate buffered saline (PBS) and incubated for 30 min at room temperature with peroxidase complex (Histofine Simple Stain MAX-PO (M); Nichirei). After sections were washed again with PBS, 3,3′-diaminobenzidine tetrahydrochloride (Nichirei) was used for color development, then sections were counterstained with hematoxylin and observed under a microscope.

Measurement of TRX levels. Saliva samples were collected using the 10-min chewing gum method. TRX levels in plasma and saliva were measured using a sandwich-type ELISA kit (Redox Bio Science, Kyoto, Japan)[22,23]. The HSG cell line established from salivary epithelial cancer cells was kindly donated by Dr. Mitsunobu Sato of Tokushima University. Cells (5×10^5/well) were seeded into 6-well dishes and then incubated 24 h in the presence of 0, 50, and 100 µM hydrogen peroxide, and then TRX levels in the supernatant were measured using the ELISA kit.

IL-6 detection by ELISA and reverse transcription-polymerase chain reaction (RT-PCR). HSG cells (3×10^5/well) were incubated 24 h with 0.1 or 1.0 µg/ml human rTRX. The medium was then discarded, and the cells were incubated an additional 24 h in fresh medium containing 500 U/ml IFN-γ (R&D Systems, Minneapolis, MN, USA). The supernatants were then collected, and IL-6 levels were measured using an ELISA kit (Endogen, Rockford, IL, USA). Alternatively, expression levels of IL-6 mRNA in HSG cells were analyzed after incubation with 500 U/ml IFN-γ for 1 h. Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and cDNA synthesis was carried out using a Script III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). Each PCR was run in duplicate using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) with the Hs00174131_A1 primer pairs according to the manufacturer's instructions. The amplification protocols and data acquisition and analysis were carried out using an ABI Prism 7900 HT instrument (Applied Biosystems). PCR was also carried out using Hs99999905_A1 primer pairs (Applied Biosystems) to amplify GAPDH, against which all samples were normalized to control for differences in the amount of total RNA added to each cDNA reaction and for variations in the reverse transcriptase efficacy among the different cDNA reactions.

Apoptosis assay. HSG cells (3×10^5/well) were incubated 24 h with 1.0 µg/ml human rTRX. The medium was discarded, and the cells were incubated an additional 24 h in medium (1 ml/well) containing 500 U/ml IFN-γ together with rTRX (1.0 µg/ml). Then the medium was discarded again and medium containing anti-Fas antibody (500 ng/ml; Medical and Biological Laboratories, Nagoya, Japan) was added for 4 h to induce apoptosis. For apoptosis detection, cells were harvested and then incubated with annexin V-FITC and PI (Roche, Basel, Switzerland) in darkness for 30 min. Samples were analyzed on a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA).

Statistical analysis. Data are presented as means ± SD of the indicated samples. Comparative statistical analyses of 2 independent groups were performed by Mann-Whitney U-test. Values of $p < 0.05$ were considered significant.

RESULTS

Production of 8-OHdG, 4-HNE, and nitrotyrosine in SS salivary glands. We examined the histological distribution of 3 determinants of oxidative stress, 8-OHdG, 4-HNE, and nitrotyrosine, in specimens of salivary gland from 5 SS patients and 2 healthy controls. The oxidative stresses against nucleotides, lipids, and proteins were detected by 8-OHdG, 4-HNE, and nitrotyrosine, respectively. Whereas little if any 8-OHdG, 4-HNE, and nitrotyrosine was detected in salivary tissues from control subjects (Figure 1G, 1H, 1I), significant production of 8-OHdG, 4-HNE, and nitrotyrosine was detected in salivary duct cells in all 5 SS tissue specimens examined. Notably, production of these 3 oxidative stress markers was detected not only in ducts within regions showing periductal lymphocytic infiltration (Figure 1A, 1B, 1C), but also in those regions without lymphocytic infiltration (Figure 1D, 1E, 1F). In addition, these markers were also detected in serous-type salivary acinar cells in all 5 SS tissue specimens examined.

Expression of TRX in salivary glands from SS patients. We

Table 1. Laboratory data from patients with SS. TRX expression in acinar cells was classified as follows: no expression, 0; < 50% of acinar cells, 1; > 50% of acinar cells, 2.

Patient, age, sex	SFR, ml/10 min	sTRX, ng/ ml	pTRX, ng/ml	Greenspan Focus Score	TRX Expression, Acinar Cell	TRX Expression, Ductral Cell
50 F	4.8	401	17.1	2	0	+
28 F	14	707	14.2	3	0	+
56 F	2	912	16.6	4	2+	+
70 F	4	1013	18.8	1	1+	+
57 F	5	1044	14	3	0	+
31 F	11	1046	30.4	4	1+	+
32 F	6.5	1083	32.3	4	1+	+
72 F	4	1640	26.8	2	0	+
48 F	11	1810	26.8	2	2+	+
61 F	5.8	2260	10.4	4	0	+
55 F	7.4	2586	15.1	3	0	+
63 F	3.1	4800	19.9	3	2+	+
30 F	5	5805	26.4	4	2+	+
53 F	3	7900	47.7	4	2+	+
31 F	4.5	9505	87	4	2+	+
65 F	5	10360	Not tested	2	1+	+
70 F	5	10400	48.1	2	1+	+
55 F	1.5	12370	90	2	1+	+
33 F	2.2	15620	25.2	4	2+	+

TRX: thioredoxin, SFR: salivary flow rate, sTRX: salivary TRX, pTRX: plasma TRX.

next carried out immunohistochemical analysis of TRX expression in salivary glands from 19 SS patients, and 2 healthy donors and 3 non-SS patients with sicca symptoms. We found that TRX was expressed in ductal cells in all 19 specimens from SS patients (Figure 2B, 2E), and its expression paralleled the production of 8-OHdG (Figure 2A, 2D). As with 8-OHdG, TRX was detected in ductal cells in regions with (Figure 2E) and without (Figure 2B) periductal lymphocytic infiltration, as well as in serous-type acinar cells in 13 of the 19 SS specimens tested. TRX expression in acinar cells was not correlated with the focus score grading lymphocytic infiltration according to the Greenspan classification (data not shown), although most of the infiltrating mononuclear cells expressed TRX. In the specimens from 2 healthy donors and 3 non-SS patients with sicca symptoms, HLA-DR was not expressed in ductal or in acinar cells (Figure 2I). In those specimens, there was only faint 8-OHdG expression and no TRX expression in ductal cells, and little or no expression of either in the acinar cells (Figure 2G, 2H). The distribution of TRX expression in the salivary tissues of SS patients was thus well correlated with that of 8-OHdG production, indicating that TRX expression reflects the local oxidative stress in SS tissues.

We next compared salivary TRX expression to HLA-DR expression and the degree of labial tissue destruction. In contrast to TRX, no HLA-DR was detected in ductal cells lacking periductal lymphocytic infiltration (Figure 2C). However, TRX was strongly expressed in ductal cells without periductal lymphocytic infiltration as well as in those with tissue destruction resulting from lymphocyte infiltration or fibrosis. This difference in the distributions of TRX and HLA-DR expression suggests the former is not directly related to inflammation.

TRX levels in saliva from SS patients. Laboratory data from SS patients are summarized in Table 1. Levels of TRX in saliva samples from SS patients were significantly higher than those from RA patients, SLE patients, and healthy controls (5441 ± 6443 ng/ml vs 779 ± 205, 1134 ± 772, and 692 ± 407 ng/ml, respectively; $p < 0.05$; Figure 3A). In addition, to verify that TRX was specifically induced in the salivary glands of SS patients, we normalized TRX levels to those of salivary albumin and found that relative levels of TRX were also significantly higher in saliva from SS patients than in control saliva from RA patients, SLE patients, and healthy subjects (Figure 3B). This is the first report that describes high TRX concentration in saliva from SS. However, salivary TRX levels were not correlated with the score according to the modified Greenspan classification[24]. In addition, TRX levels were about 10-fold higher in the saliva of healthy subjects than in their plasma (749.9 ± 414.11 vs 53.4 ± 14.22 ng/ml; n = 10).

Salivary flow rates are inversely related to salivary TRX levels. When we analyzed the relationship between salivary flow rates and TRX levels in SS patients, we found that salivary flow rate was < 10 ml/10 min (the lower limit of healthy subjects) in 16 of the 19 SS patients, and that it correlated inversely with the salivary TRX level ($r = –0.464$, $p < 0.02$; Figure 3C). As the salivary flow rate is one index of salivary function, this inverse relation suggests that the TRX level reflects the clinical severity of the SS. By contrast, salivary flow rate was not correlated with salivary levels of either IFN-

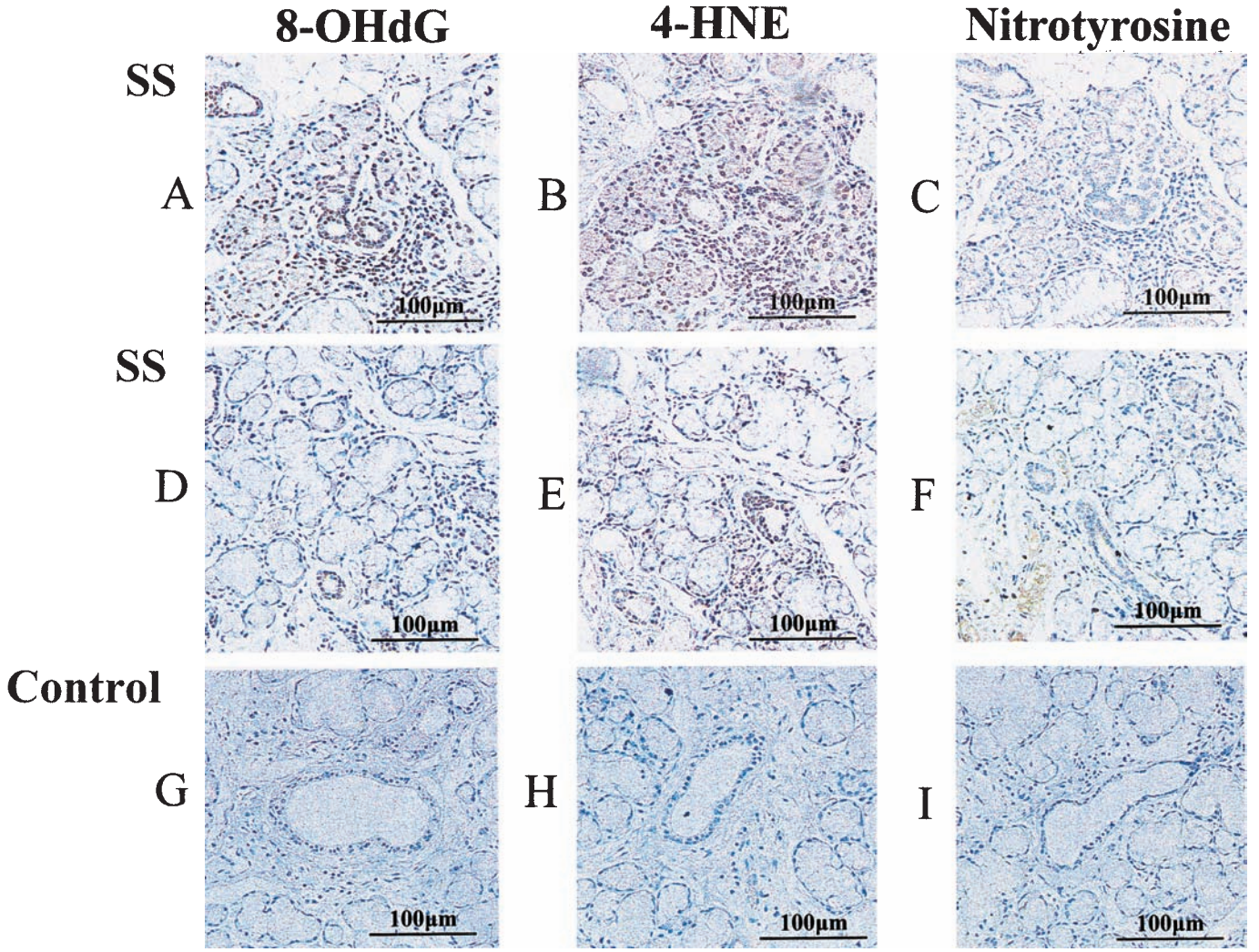

Figure 1. Ductal epithelial cells in salivary tissues from SS patients produce large amounts of 8-OHdG, 4-HNE, and nitrotyrosine, detected in ducts with (A, B, C) and without (D, E, F) periductal lymphocytic infiltration.

γ or IL-6 (data not shown). In addition, there was a significant correlation between plasma and salivary TRX (Figure 3D).

Salivary TRX levels are markedly increased in SS patients showing acinar TRX expression. When SS patients were divided into 2 groups based on whether they exhibited acinar cell TRX expression or not, we found that the mean salivary TRX concentration was significantly higher in patients who showed acinar TRX expression than in those who did not (6355.7 ± 1396.5 ng/ml vs 1439 ± 871 ng/ml; $p < 0.05$; Figure 4). TRX expression in acinar cells was confirmed in salivary specimens from 7 SS patients whose salivary TRX levels exceeded 4000 ng/ml. Irrespective of whether they exhibited acinar TRX expression, however, salivary TRX levels in SS patients were always significantly higher than in controls ($p < 0.05$).

Statistical analysis revealed that the Greenspan grade was not correlated with salivary TRX concentration or acinar TRX expression.

Oxidative stress induces TRX in HSG cells. The parallel production of 8-OHdG and TRX in ducts without HLA-DR expression suggests that TRX expression might be induced by oxidative stress rather than by local inflammation. We therefore tested whether TRX could be induced by a typical oxidant, H_2O_2, in the HSG cell line. Using a specific ELISA, we found that incubating the cells with H_2O_2 increased TRX levels from 9.77 ± 3.84 ng/ml to 41.16 ± 16.20 ng/ml (H_2O_2 50 μM) or 166.01 ± 47.14 pg/ml (H_2O_2 100 μM) in a time- and dose-dependent manner (Figure 5A).

TRX suppresses IL-6 production in HSG cells. To further clarify the role of TRX in SS, we next examined the effect of rTRX on the production of a representative inflammatory cytokine (IL-6) in HSG cells. We found that pretreating the cells with rTRX reduced IFN-γ-induced IL-6 production from 358.13 ± 19.73 pg/ml to 235.21 ± 9.53 pg/ml (TRX 0.1 μg/ml) or 193.68 ± 15.71 pg/ml (TRX 1.0 μg/ml) in a time- and dose-dependent manner (Figure 5B). Interestingly, pretreatment with rTRX also reduced spontaneous IL-6 secretion from

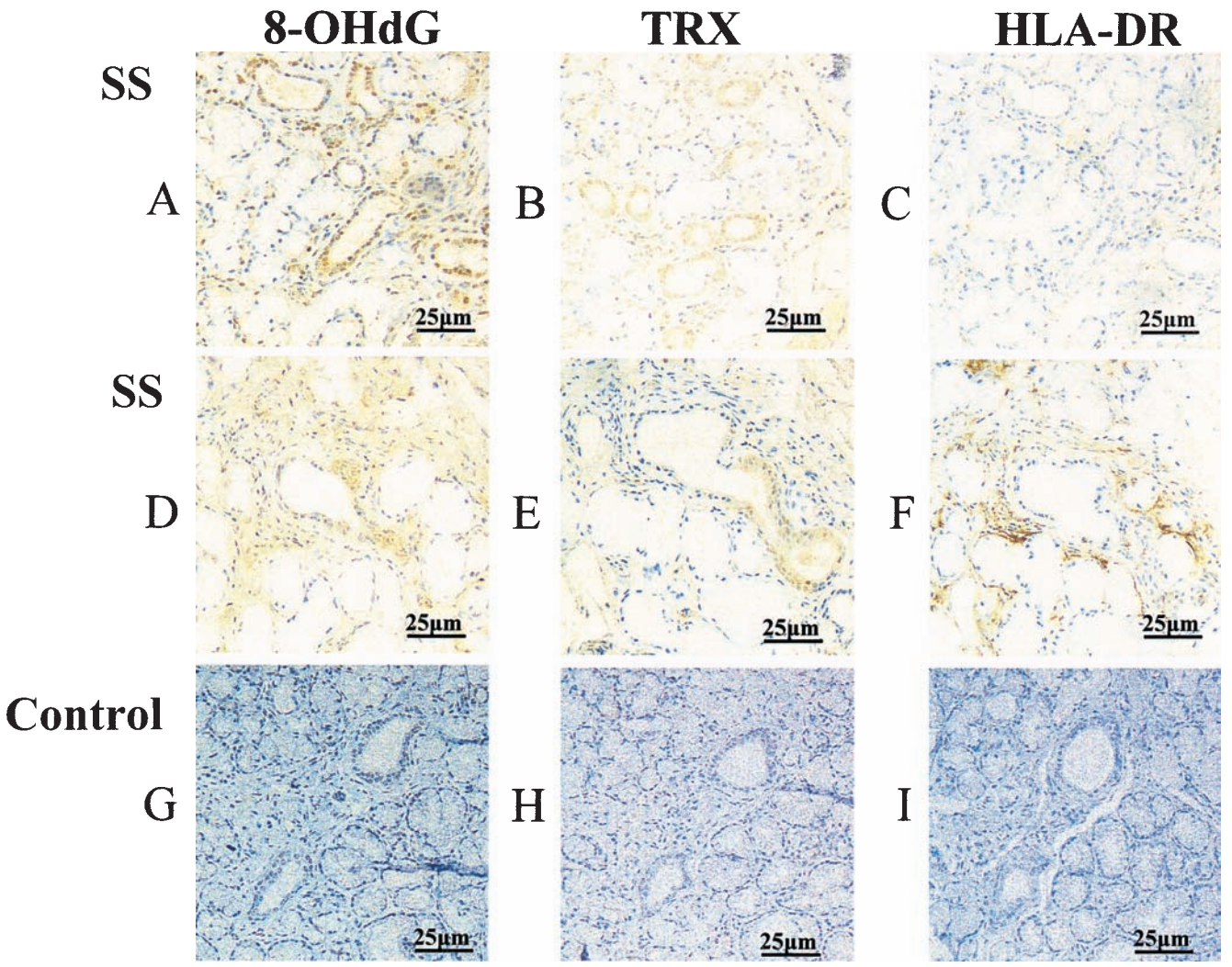

Figure 2. TRX and 8-OHdG are produced in ductal cells without periductal lymphocytic infiltration and HLA-DR expression (A, B, C), as well as in those with massive lymphocytic infiltration (D, E, F) in SS patients. In non-SS patients with sicca symptoms, there was no expression of HLA-DR (I). Only faint expression of 8-OHdG and no expression of TRX were detected in ductal cells, and little or no expression of both in acinar cells (G, H).

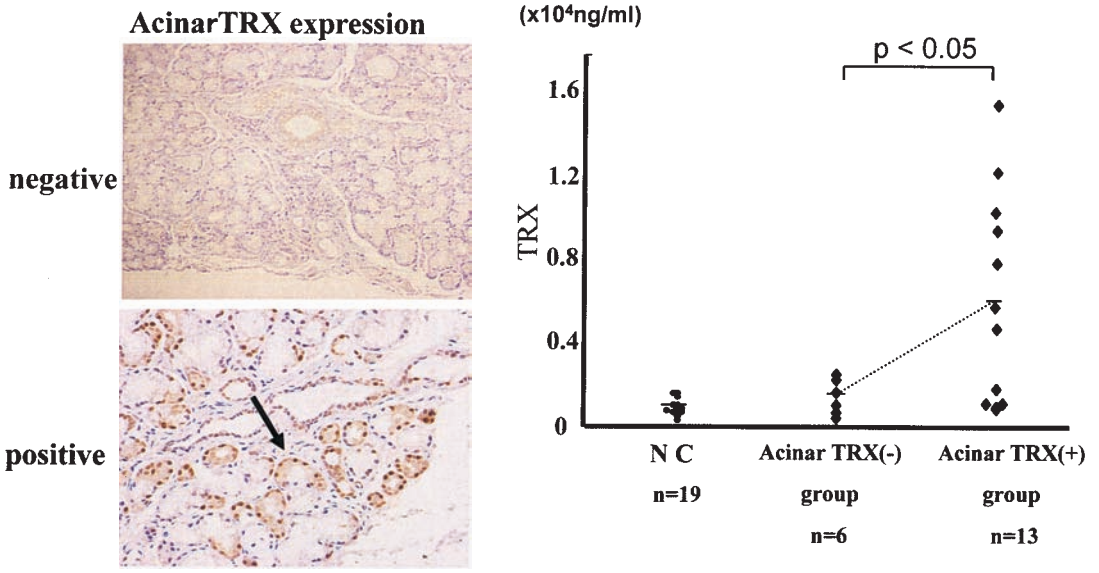

Figure 4. TRX expression in salivary tissues from SS patients. Concentration of salivary TRX was significantly higher in patients who showed acinar TRX expression than in those who did not. NC: normal controls.

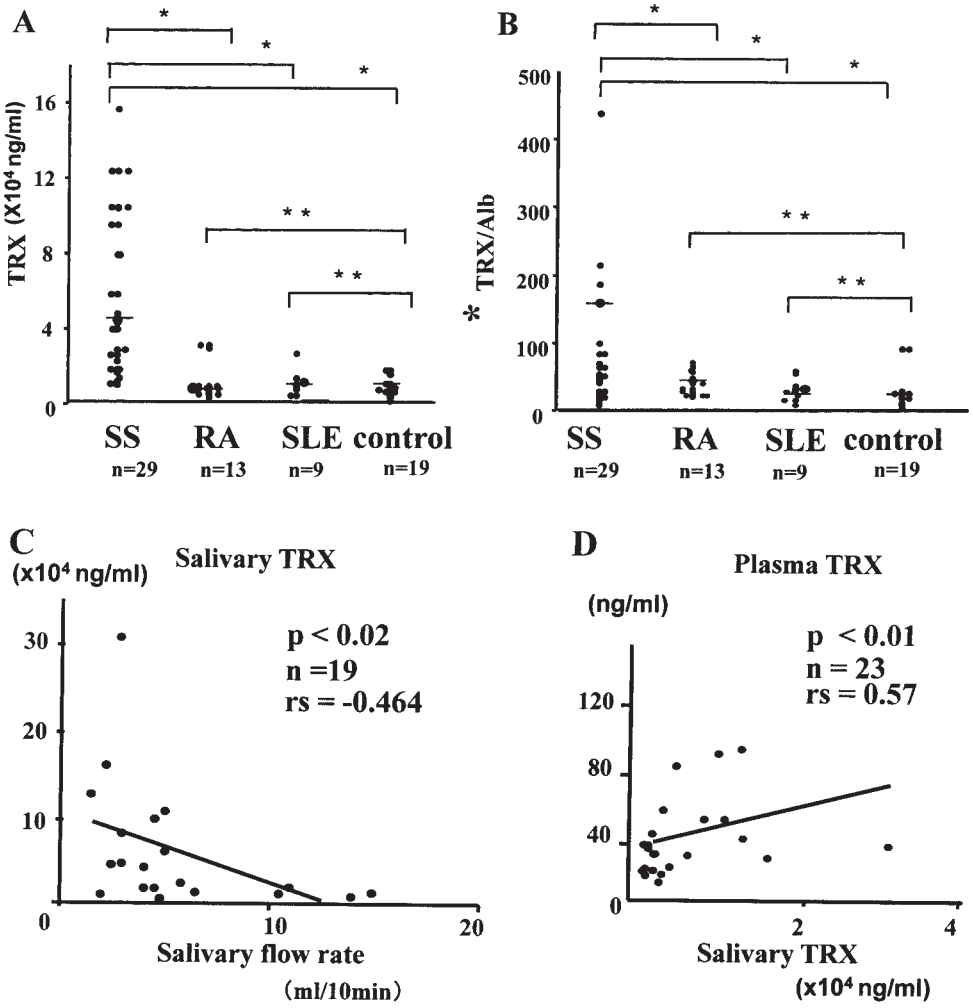

Figure 3. Salivary TRX levels in SS patients and correlation between plasma and salivary TRX levels in SS patients. (A) TRX levels in salivary specimens from 29 SS patients, 13 RA patients, 9 SLE patients, and 19 healthy controls were measured by ELISA and found to be significantly higher in the former ($p < 0.05$) *$p < 0.05$; **nonsignificant. (B) *TRX levels were normalized by dividing them by salivary albumin levels. *$p < 0.05$; **nonsignificant. (C) In addition, there was significant inverse correlation between salivary flow rates and TRX levels in SS patients. (D) A significant correlation was found between plasma and salivary TRX levels ($p < 0.01$).

HSG cells from 166.07 ± 33.4 pg/ml to 121.06 ± 37.24 pg/ml. In addition, as the level of IL-6 mRNA peaked after about 1 h of IFN-γ stimulation (data not shown), we examined the IL-6 mRNA level after 1 h of incubation with IFN-γ. We found that pretreating the cells with rTRX significantly attenuated IFN-γ-induced IL-6 transcription (Figure 5C).

TRX suppresses Fas-mediated apoptosis in HSG cells. Finally, we used annexin V and PI staining to test whether rTRX affects the Fas-mediated apoptosis in HSG cells or not. Since undesirable cell-surface expression of autoantigens on ductal cells, whose expression is postulated to trigger sialoadenitis of SS, is caused by Fas-mediated apoptosis[25]. HSG cells were induced to undergo apoptosis by anti-Fas antibody after IFN-γ treatment. Anti-Fas antibody induced fewer annexin V-positive cells with rTRX pretreatment than without pretreatment, meaning that HSG cells were less apoptotic when pretreated with rTRX (Figure 5D).

DISCUSSION

In contrast to other chronic liver diseases, such as viral and autoimmune hepatitis, in which 8-OHdG is predominantly produced in hepatocytes, 8-OHdG is produced in the bile ducts of patients with primary biliary cirrhosis (PBC)[26]. Notably, PBC and SS often occur together in the same patients, and both are characterized by periductal lymphocytic infiltration. Consequently, expression of 8-OHdG in ductal cells is regarded as a common denominator for both diseases. There have been several reports analyzing the role of ductal cells in the pathogenesis of SS. These studies showed (1) that T cell-attracting chemokines CXCL9 and IP10 (CXCL10)[27],

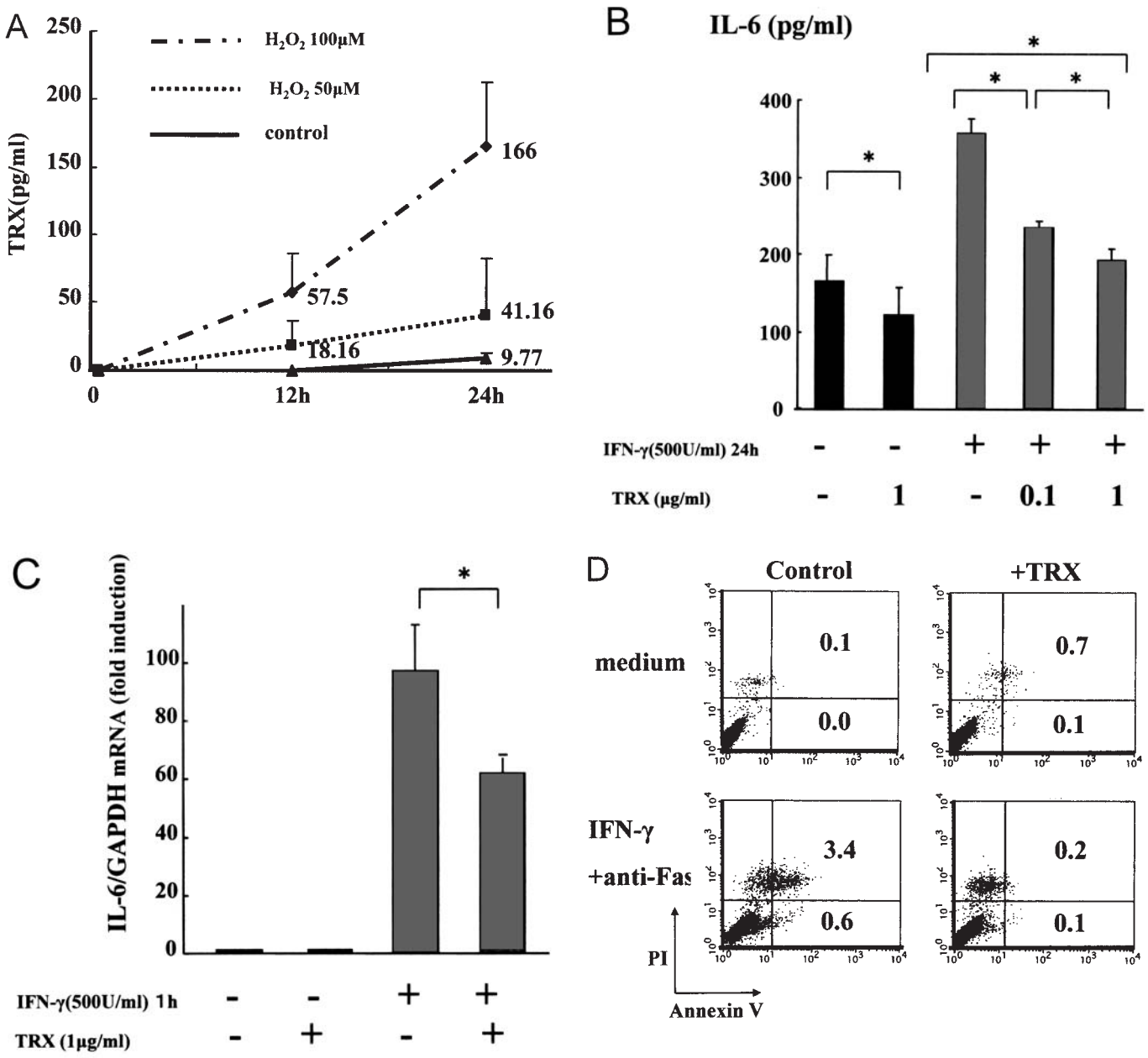

Figure 5. Suppression of IL-6 production and Fas-mediated apoptosis in HSG cells by TRX pretreatment. (A) Incubation with H_2O_2 increased TRX secretion from HSG cells in a time- and dose-dependent manner (ELISA). (B) Pretreatment with rTRX for 24 h reduced IFN-γ-induced IL-6 secretion from HSG cells in a time- and dose-dependent manner (ELISA). *$p < 0.05$. (C) Pretreatment with rTRX substantially reduced IFN-γ-induced expression of IL-6 mRNA in HSG cells. Production of IL-6 mRNA was analyzed by quantitative RT-PCR. *$p < 0.01$. (D) Pretreatment with rTRX reduced Fas-induced apoptosis in HSG cells. HSG cells were stained with annexin V and PI, and apoptotic cells were detected by FACScan. Representative data from 3 independent experiments are shown in each figure.

B cell-attracting chemokines CXCL12 and CXCL13[28] and monokines induced by IFN-γ (CXCL9) are predominantly expressed in ductal cells[27]; (2) that NFS/sld mutant mice, a murine model of SS, express α-fodrin, a candidate autoantigen in SS, in salivary ductal cells[29,30]; and (3) that epithelial cells from SS patients show 40-fold higher levels of IL-1α, IL-6, TNF-α, and IFN-γ mRNA than control cells[31]. It is therefore interesting that ductal cells in particular are under strong oxidative stress in SS.

Our findings also demonstrate that salivary ductal cells from SS patients express high levels of TRX. Moreover, the colocalization of TRX and 8-OHdG in ductal cells indicates that TRX expression is induced by oxidative stress. Notably, TRX expression was observed in ductal cells from SS patients with no sign of a local inflammation as well as in those showing massive periductal lymphocyte infiltration. Therefore, the

extent to which TRX affects periductal lymphocytic infiltration remains to be determined. However, in addition to eliminating oxidative stress, TRX is known to exert antiinflammatory and antiapoptotic effects[18]. For example, TRX reportedly inhibits lipopolysaccharide-induced IL-1ß expression[32] and negatively regulates TNF-α-induced MAP kinase activation and IL-6 production[33]. TRX also may attenuate neutrophilic infiltration into sites of inflammation[34]. Thus, TRX expression in ductal cells may function as an immunological modulator of local inflammation. Consistent with that idea, our findings show that pretreating HSG cells with rTRX reduced IL-6 secretion in both the presence and absence of IFN-γ. Moreover, rTRX treatment negatively regulated IFN-γ-induced IL-6 transcription in HSG cells. That TRX is known to act as a transcription factor suggests it may act directly on the promoter region of IL-6 gene. In addition, we also observed that rTRX suppressed Fas-mediated apoptosis under the existence of IFN-γ. These results suggest that TRX may play both antiinflammatory and antiapoptotic roles in salivary ductal cells to protect them from various types of stresses at the early phase of SS. Our results are in accord with reports that TRX plays protective roles in murine models of anti-collagen type II antibody-induced arthritis and bleomycin-induced interstitial lung disease[35,36]. Interestingly, TRX had no effect on IFN-γ-induced expression of the cell surface antigens HLA-DR and intercellular adhesion molecule-1, which suggests it may selectively regulate the IFN-γ signaling pathway in ductal cells.

Apparently, salivary acinar cells produced large amounts of TRX in SS. Similarly, thyroid follicular cells affected by Graves' disease also express TRX[37], and TRX reportedly exerts a growth-promoting effect on regenerating Paneth cells in rat intestine[38]. These findings, together with our observation that TRX levels were 10-fold higher in saliva than in plasma in healthy subjects, suggest salivary acinar cells may be natural TRX secretors that respond to oxidative stress, and the high salivary TRX levels observed in SS reflect their destruction or regeneration due to exposure to potent oxidative stress.

Finally, salivary flow rates were inversely related to the salivary TRX concentration in SS patients. This means that elevation of salivary TRX levels may reflect the SS disease activity, making TRX a useful biomarker for assessing SS activity.

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