Estrogen Deficiency Accelerates Autoimmune Exocrinopathy in Murine Sjögren's Syndrome through Fas-Mediated Apoptosis

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Estrogenic action has been suggested to be responsible for the strong female preponderance of autoimmune diseases, but the role of estrogens in the female has not been well characterized. We evaluated the effects of estrogen deficiency in a murine model for autoimmune exocrinopathy of Sjögren's syndrome (SS). Severe destructive autoimmune lesions developed in the salivary and lacrimal glands in estrogen-deficient mice, and these lesions were recovered by estrogen administration. We detected an intense estrogen receptor in splenic CD8⁺ T cells compared with that in CD4⁺ T cells, and concanavalin-A-stimulated blastogenesis of splenic CD8⁺ T cells with estrogens was much higher than that of CD4⁺ T cells. We found a significant increase in serum autoantibody production against the organ-specific autoantigen α -fodrin. Moreover, an increased proportion of TUNEL⁺ apoptotic epithelial duct cells was observed in estrogen-deficient mice. It was demonstrated that Fas-mediated apoptosis in cultured salivary gland cells was clearly inhibited by estrogens *in vitro*. These results indicate that dysfunction of regulatory T cells by estrogen deficiency may play a crucial role on acceleration of organ-specific autoimmune lesions, and estrogenic action further influences target epithelial cells through Fas-mediated apoptosis in a murine model for SS. (Am J Pathol 1999, 155:173-181)

Sex hormones influence both humoral and cell-mediated immune response, and estrogen is one of the potential factors in this immunological dimorphism.¹⁻⁴ Estrogenic action has been suggested to be responsible for the strong female preponderance of autoimmune diseases, including systemic lupus erythematosus (SLE), scleroderma, and Sjögren's syndrome (SS).⁵⁻⁸ Both estrogen and testosterone have important roles in the development of autoimmune lesions, and they also modulate immune responses in a number of animal models.⁹⁻¹¹

SS in humans is an organ-specific autoimmune disease characterized by lymphocytic infiltration into the salivary and lacrimal glands, resulting in symptoms of dry mouth and dry eye due to insufficient secretion.¹² Several animal models for investigating human SS are known to occur spontaneously in autoimmune-prone mice such as NZB/NZWF1 and MRL/*lpr* strain¹³⁻¹⁵ and in non-autoimmune-prone NFS/*sld* mice thymectomized 3 days after birth.¹⁶ It is possible that individual T cells activated by an appropriate antigen can proliferate and form a restricted clone.^{17,18} Recently, we identified 120-kd α -fodrin as an important autoantigen in both NFS/*sld* murine model for SS and human SS patients.¹⁹ On the other hand, it has been recently demonstrated that the Fas-FasL system plays a major role on the induction of apoptosis in target organs with autoimmune diseases such as autoimmune gastritis, Hashimoto's thyroiditis, and rheumatoid arthritis (RA).²⁰⁻²⁴ Since it was reported that Fas expression was observed in the salivary gland cells with human SS,²⁵ we speculate that Fas-mediated apoptosis may contribute to tissue destruction in the salivary glands with SS. The *in vivo* roles of estrogens for tissue destruction through Fas-mediated apoptosis in autoimmune lesions has not yet been analyzed. We report here that estrogen deficiency induced by ovariectomy (Ovx) accelerates destructive autoimmune lesions, and these lesions were recovered by estrogen administration in a murine SS model. We analyzed the effects of estrogen deficiency in this model from various approaches, including Fas-mediated apoptosis toward target tissue destruction.

Materials and Methods

Mice and Treatment

NFS/*sld* mice carrying the mutant gene *sld*²⁶ were bred in our own facilities maintained in a specific-pathogen-free mouse colony and given food and water *ad libitum*. Thymectomy (Tx) was performed on day 3 after birth, and then female mice 4 weeks of age were ovariectomized

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(Ovx) and compared with sham-operated (non-Ovx), non-Tx, and Tx alone female NFS/*sld* mice. Five to seven mice in each group were analyzed at 8, 12, 16, and 20 weeks of age. Tx plus Ovx mice were intramuscularly administered with 60 mg/kg/week estrogen (Ovahormone depo; Teikoku Zouki, Tokyo, Japan) in sesame oil or subcutaneously 25 mg/kg/day testosterone (Wako Pure Chemical, Osaka, Japan) and 2.5 mg/kg/day tamoxifen (RBI, MA) in olive oil from 4 to 8 weeks of age. These concentrations were chosen to be the most biologically active form, as described previously.^{27–29} Administrations were performed in five groups as follows: Tx alone ($n = 18$), Tx plus Ovx ($n = 6$), Tx plus Ovx plus estrogen ($n = 5$), Tx plus tamoxifen ($n = 5$), and Tx plus Ovx plus testosterone ($n = 5$).

Histopathology

All organs were removed from the mice, fixed with 10% phosphate-buffered formalin, and embedded in paraffin. The sections (4 μ m) were stained with hematoxylin and eosin. Histological grading of inflammatory lesions was done according to the method proposed by White and Casarett³⁰ as follows: score 1 indicates that 1 to 5 foci being composed of more than 20 mononuclear cells per focus were seen, score 2 indicates that more than 5 such foci were seen but without significant parenchymal destruction, score 3 indicates that degeneration of parenchymal tissue, and score 4 indicates extensive infiltration of the glands with mononuclear cells and extensive parenchymal destruction.

In Situ End Labeling of Fragmented DNA (TUNEL)

Apoptotic cells were detected in sections using the *in situ* TUNEL kit (Wako Pure Chemical), as described previously.³¹ Briefly, paraffin-embedded sections were deparaffinized, rehydrated, and washed twice in phosphate-buffered saline (PBS). Sections were incubated with proteinase K (20 μ g/ml) for 10 minutes. After washing in distilled water, these sections were incubated with 2% H₂O₂ in PBS to block endogenous peroxidase. Sections were then presoaked in TdT buffer (0.5 mmol/L cacodylate, 1 mmol/L CoCl₂, 0.5 mmol/L dithiothreitol, 0.05% bovine serum albumin, 0.15 mol/L NaCl) for 10 minutes, and incubated for 2 hours at 37°C in 25 μ l of TdT solution, containing 1X terminal transferase buffer, 0.5 nmol of biotin-dUTP, and 10 U of TdT (WAKO). After the TdT reaction, sections were soaked in TdT blocking buffer (300 nmol/L NaCl, 30 mmol/L trisodium citrate-2-hydrate), incubated with horseradish-peroxidase-conjugated streptavidin for 30 minutes at room temperature, and developed for 10 minutes in phosphate-buffered citrate (pH 5.8) containing 0.6 mg/ml diaminobenzidine. Nuclei were counterstained with hematoxylin. When we used DNase-I-treated and untreated sections of submandibular glands in non-Tx mice, almost all acinar and duct cells were TUNEL⁺ in DNase-I-treated sections and were TUNEL[−] in untreated sections (data not shown).

Flow Cytometry

Spleen cell suspensions were stained with antibodies conjugated to phycoerythrin (anti-CD4, Cedar Lane Laboratories, Ontario, Canada; B220, Pharmingen, San Diego, CA) and fluorescein isothiocyanate (anti-CD8, Cedar Lane Laboratories; Thy1.2, anti-I-A^s, anti-CD5, Pharmingen) and analyzed with FACScan (Becton Dickinson, Mountain View, CA). Isolated mouse salivary gland cells (described below) were stained with biotinylated anti-Fas monoclonal antibody (MAb; Pharmingen) and fluorescein-isothiocyanate-conjugated avidin (Vector Laboratories, Burlingame, CA) and analyzed by FACS.

Cell Preparation

Spleen cells were aseptically prepared from 8- to 10-week-old, untreated NFS/*sld* female mice. To purify CD4 or CD8 single-positive T cells, we used nylon fiber for T cell selection and immunomagnetic beads (DynaI, CA) with anti-CD4 MAb, anti-CD8 MAb, and Mac-1 MAb (Becton Dickinson, San Jose, CA) as reported previously.^{32,33} The purity of CD4 or CD8 single-positive cells was 90% or more. We further obtained tissue-infiltrating mononuclear cells in the salivary gland as reported previously.³⁴ Briefly, the inflamed submandibular glands from 8- to 10-month-old Tx plus Ovx and Tx alone mice were removed, cut into small pieces with scissors through 100-gauge stainless steel mesh, and suspended in RPMI 1640 containing 10% fetal calf serum, 10 mmol/L HEPES buffer, penicillin (100 U/ml), and streptomycin (100 μ g/ml). After washing twice with the medium, infiltrating mononuclear cells were isolated from parenchymal cells by Ficoll-Isopaque density (1090) gradient centrifugation.

Proliferation Assay

Single-cell suspensions of spleen cells from treated and untreated NFS/*sld* mice were cultured in 96-well flat-bottom microtiter plates (5 $\times$ 10⁵ cells/well) in RPMI 1640 containing 10% fetal calf serum, penicillin/streptomycin, and β -mercaptoethanol. Cells were cultured with 10^{−9} mol/L β -estradiol (E2; Wako Pure Chemical), 10^{−9} mol/L tamoxifen (Tam; RBI), 2.0 μ g/ml concanavalin (Con)A (EY Laboratories, San Mateo, CA), and 4.0 μ g/ml lipopolysaccharide (LPS; DIFCO, Detroit, MI). [³H]Thymidine incorporation during the last 20 hours of the culture was evaluated using an automated β liquid scintillation counter.

Western Blot Analysis

To detect estrogen receptor (ER) in cytosol of CD4 or CD8 single-positive splenocytes, lysis of cells was performed using lysis buffer (2 μ g/ml aprotinin, 1 μ mol/L EDTA). Briefly, after addition of 1 ml of lysis buffer, the lysate was incubated on ice for 10 minutes and centrifuged at 10,000 rpm for 30 minutes, and the supernatant was immediately analyzed for each experiment. Western blot analysis was performed using anti-human estrogen

receptor MAb (Transduction Laboratories, Lexington, KY; cross-reactivity with mouse estrogen was confirmed). To detect serum autoantibodies against salivary-gland-specific 120-kd α -fodrin antigen,¹⁹ the autoantigen was electrophoresed in nonreducing buffer in 10% SDS-polyacrylamide gel electrophoresis gels, and the protein was then electrophoretically transferred to nitrocellulose, which was probed with serum antibodies from untreated, Tx, and Tx plus Ovx mice. Nitrocellulose membranes were incubated with peroxidase-conjugated horse anti-mouse IgG (Vector Laboratories). Autoantibodies were detected using ECL Western blotting reagent (Amersham Corp., Arlington, IL).

ELISA

Serum autoantibodies were detected using recombinant α -fodrin protein (JS-1).¹⁹ After coating with the recombinant α -fodrin protein in a 96-well ELISA plate, biotinylated anti-mouse IgG (Vector Laboratories) was added as second Ab. Measurements of JS-1-specific autoantibodies were read by an automatic ELISA reader (Flow Laboratories, McLean, VA). To detect serum antibody to DNA, the relative avidity of the serum antibodies for binding to single-stranded (ss)DNA was measured in a solid-phase ELISA. Purified ssDNA was prepared by boiling calf thymus DNA (Sigma Chemical Co., St. Louis, MO) for 10 minutes followed immediately by dilution in ice-cold borate-buffered saline. Flat-bottom 96-well microtiter plates (Nunc, Denmark) were coated with ssDNA (50 μ g/ml) overnight at 4°C in PBS, blocked at room temperature with 5% skim milk, and incubated with sera. Plates were read on an automatic ELISA reader (Flow Laboratories) at 492 nm.

Primary Culture of Mouse Salivary Gland Cells

Mouse salivary gland epithelial cells were prepared as previously described.^{35,36} Mouse salivary glands were taken from five female NFS/*sld* mice (3w), decapsulated, minced into 1-mm² pieces, washed with Hanks' balanced salt solution without Ca²⁺ and Mg²⁺, and placed in a 60-dish culture plate containing HBSS with 0.76 μ g/ml EDTA, 4.9 μ g/ml L-ascorbic acid, and 4.9 μ g/ml reduced glutathione. This mixture of solution was placed in a shaking water bath at 37°C for 15 minutes. Fragments were washed with Dulbecco's modified Eagle's medium/STI, and placed in a mixture of collagenase (type I, 750 U/ml) and hyaluronidase (type IV, 500 U/ml) dissolved in Dulbecco's modified Eagle's medium/F12 containing 10% fetal calf serum. The first digest suspension was passed through sterile 100- μ m nylon mesh filter and redigested for 30 minutes by the same digestion procedure, and then the digest suspension was passed through a 100- μ m nylon mesh filter. Adherent cells after culture in minimal essential medium containing 10% fetal calf serum for 24 hours at 37°C were isolated as salivary gland epithelial cells. We confirmed that the cells over 95% were positively stained with anti-keratin polyclonal antibody. Mouse salivary gland cells were cultured with

Figure 1. Mean grade of autoimmune lesions in the salivary and lacrimal glands in estrogen-deficient NFS/*sld* animal model (Tx+Ovx) compared with Tx alone mice. Ovx was performed at 4 weeks of age, and histological features of each gland were examined at 8, 12, 16, and 20 weeks of age. Grading of inflammatory lesions was classified according to the method of White and Casarett.³⁰ **P* < 0.01; ***P* < 0.001, Mann-Whitney U test.

1000 U/ml interferon (IFN)- γ (Pharmingen) for 24 hours, and apoptosis was induced by 100 ng/ml anti-Fas MAb (Pharmingen) in the absence or presence of estrogens for an additional 24 hours. Cells were harvested by centrifugation and analyzed for apoptotic nuclei with propidium iodide.

Results

Pathology of Tx plus Ovx Mice

Treatment of female NFS/*sld* mice with thymectomy (Tx) 3 days after birth has been shown to induce autoimmune lesions exclusively in the salivary and lacrimal glands as described in detail.¹⁶ To examine the *in vivo* effects of estrogen deficiency in these mice, ovariectomy (Ovx) was performed at 4 weeks when these inflammatory lesions begin to develop. Unexpectedly, severe destructive autoimmune lesions developed in the salivary (submandibular, parotid, and sublingual) and lacrimal glands in Tx plus Ovx mice compared with those in Tx alone female mice (Figure 1). Multiple confluent foci of mononuclear cell infiltration with parenchymal destruction were seen in Tx plus Ovx mice. No inflammatory lesions were seen in any other organ, and no autoimmune lesions were observed in Ovx alone NFS/*sld* mice until 20 weeks of age. These findings indicate that an estrogen defi-

Figure 2. Effects of administration with estrogen (E2), tamoxifen (Tam), and testosterone (Tes) in estrogen-deficient NFS/*sld* animal model (Tx+Ovx). Severe autoimmune lesions in Tx+Ovx mice were recovered by the intramuscular administration with estrogen at 8 weeks of age. Testosterone administration in Tx+Ovx mice induced severe inflammatory lesions compared with those in Tx+Ovx mice. Grading of inflammatory lesions was classified according to the method of White and Casarett.³⁰ * $P < 0.05$; ** $P < 0.005$, Mann-Whitney U test.

ciency in Tx mice could be attributed to severe destructive autoimmune lesions in the salivary and lacrimal glands.

Recovery of Severe Autoimmune Lesions by Estrogen Administration

The severe destructive autoimmune lesions in Tx plus Ovx mice were inhibited by intramuscular estrogen administration as observed at 8 weeks of age (Figure 2). Control mice given the vehicle (olive oil, $n = 5$) for the estrogen injection had the same histological score as those of Tx plus Ovx mice in each gland. Tamoxifen administration in Tx alone mice induced severe inflammatory lesions to the same grade of Tx plus Ovx mice, except for the effect on the submandibular glands. A possible explanation could be raised that organ sensitivity to tamoxifen is different among these glands due to a different distribution of estrogen receptor, as described previously.³⁷ Testosterone administration in Tx plus Ovx mice induced more severe inflammatory lesions compared with those in Tx plus Ovx mice (Figure 2).

Effect of an Estrogen Deficiency on T Cell Responses

To examine whether the autoimmune responses in Tx plus Ovx mice was affected by the phenotypic changes

Figure 3. Flow cytometric analysis of spleen cells and tissue-infiltrating lymphocytes in the salivary glands from Tx alone and Tx+Ovx mice. **A:** A significant decrease of CD8⁺ T cells was observed in spleen of Tx+Ovx mice. Similar results were obtained from five samples examined per group. **B:** A major proportion of tissue-infiltrating T cells in the salivary glands was CD4⁺ of both Tx alone and Tx+Ovx mice. Five samples were analyzed per group. **C:** Expression of estrogen receptor in CD8⁺ T cell subset in spleen. **a:** SDS-polyacrylamide gel(10%) electrophoresis stained with Coomassie blue. **b:** Estrogen receptor was detected in cytosol fraction of CD8⁺ spleen T cells on Western blot analysis. Positive control represents estrogen receptor in tissue homogenate from uterus. **c:** Housekeeping CD3 protein was detected in both CD4⁺ and CD8⁺ T cells on Western blot analysis.

of peripheral T cells, we analyzed the surface phenotype in spleen cells. A significant decrease of CD8⁺ T cells in Tx plus Ovx mice was observed compared with those in Tx alone mice, while no significant change of CD4⁺ T cells was found in either group (Figure 3A). The majority of tissue-infiltrating mononuclear cells was CD4⁺ T cells with a minor proportion of CD8⁺ T cells in Tx plus Ovx mice and Tx alone mice (Figure 3B). We examined the expression of estrogen receptor (ER) on protein level in the cytosol of splenic CD4⁺ and CD8⁺ T cells from non-Tx NFS/*sld* mice. A more intense expression of ER in the cytosol of CD8⁺ T cells was observed than that of CD4⁺ T cells (Figure 3C). We next examined the *in vitro* effect of estrogens in ConA-stimulated proliferation of spleen cells. A significant increase in proliferative response of whole spleen cells to ConA plus estrogen was

Figure 4. Dose-response analysis of estrogen and tamoxifen on proliferative response of spleen cells. **A:** A dose-dependent increase in proliferative response of spleen cells to ConA plus estrogen. **B:** A dose-dependent effect of tamoxifen (from 1×10^{-10} to 1×10^{-6} mol/L) was seen on the proliferative response of spleen cells to ConA plus estrogen (10^{-9} mol/L). The antagonistic effect with various concentrations of tamoxifen was observed.

observed, compared with those to ConA alone and ConA plus estrogen plus tamoxifen (Figure 4A and Table 1). We found significant responsiveness in ConA-stimulated CD8 single-positive T cells with estrogens, but not in CD4 single-positive T cells with estrogens (Table 1). Tamoxifen had an *in vitro* suppressive effect on these responses in spleen cells and CD8-single-positive cells. In this case, we confirmed the antagonistic effect with various concentrations of tamoxifen on the estrogen response of spleen cells *in vitro* (Figure 4B).

Effect of an Estrogen Deficiency on B Cell Responses

A significant increase in proliferative response to LPS was observed in Tx plus Ovx mice compared with that in Tx alone mice (Figure 5A). When we analyzed serum autoantibody production against organ-specific autoantigen on Western blotting, we found an intense band of anti-120-kd α -fodrin autoantibody in sera from Tx plus Ovx mice compared with that in Tx alone mice (Figure 5B). A higher titer of serum autoantibodies against 120-kd α -fodrin was detected in Tx plus Ovx mice as compared with Tx alone mice by ELISA (Figure 5C), whereas no significant activity of ssDNA binding was found (Figure 5D).

Effect of an Estrogen Deficiency on Fas-Mediated Apoptosis

A significant increase of TUNEL⁺ apoptotic epithelial duct cells in the salivary glands was detected in Tx plus Ovx mice compared with Tx alone and non-Tx control mice at all ages (Figure 6). We next analyzed the effects of estrogens on IFN- γ -induced-Fas expression, and Fas-mediated apoptosis of the cultured salivary gland cells from non-Tx NFS/sld mice *in vitro*. It is well known that IFN- γ up-regulates Fas expression on various mammalian cells,^{38,39} and we also found that Fas expression on these cells was up-regulated by IFN- γ (Figure 7A). Mean fluorescence intensity (MFI) of IFN- γ -induced-Fas expression was clearly reduced by estrogens, but not by tamoxifen. When we analyzed anti-Fas MAB-stimulated apoptosis of these cells in the absence or presence

Table 1. *In Vitro* Effect of Estrogens in Con-A-Stimulated Proliferative Responses of Whole Spleen Cells and Purified CD4⁺ and CD8⁺ Cells

	Spleen cells	CD4 ⁺ cells	CD8 ⁺ cells
Medium	462.3 $\pm$ 28.4	511.6 $\pm$ 62.9	562.5 $\pm$ 88.4
E2	621.3 $\pm$ 51.5	661.4 $\pm$ 49.3	482.9 $\pm$ 62.7
Con A	18452.1 $\pm$ 261.8	46254.4 $\pm$ 2216.5	54862.1 $\pm$ 1921.3
Con A + E2	34624.6 $\pm$ 1411.5*	49215.0 $\pm$ 1211.6	86211.6 $\pm$ 2961.6*
Con A + E2 + Tam	17285.9 $\pm$ 621.4	45161.7 $\pm$ 921.4	53811.9 $\pm$ 1621.8
Tam	562.3 $\pm$ 61.3	714.6 $\pm$ 89.2	618.2 $\pm$ 51.6

A significant increase in proliferative response of whole spleen cells to Con A plus estrogen was observed, compared with those to Con A alone and Con A plus estrogen (E2) plus tamoxifen (Tam). A significant increase was seen in Con-A-stimulated CD8-single-positive T cells with E2, but not in CD4-single-positive T cells with E2. Tamoxifen had a suppressive effect on these responses in whole spleen cells and CD8-single-positive cells. Data are expressed as counts per minute per culture $\pm$ SD in triplicate. Similar results were obtained in three different experiments from three to five mice per group.

* $P < 0.01$, Student's *t*-test.

Figure 5. Effects of estrogen deficiency on B-cell response and autoantibody production. **A:** LPS-stimulated proliferative response of spleen cells from Tx+Ovx, and Tx alone NFS/*sld* mice. A significant increase of LPS responsiveness was observed in Tx+Ovx mice. * $P < 0.01$, Student's *t*-test. **B:** Detection of serum autoantibodies specific for the salivary gland autoantigen, 120-kd α -fodrin, on Western blot analysis. Representative experiment demonstrates a more intense band of anti-120-kd α -fodrin in Tx+Ovx mice than that in Tx alone mice. Five sera were examined per group. **C:** A high titer of serum autoantibodies against 120-kd α -fodrin was detected in Tx+Ovx mice, compared with that in Tx alone mice by ELISA. Results indicate mean $\pm$ SEM values for five samples examined per group. * $P < 0.01$; ** $P < 0.001$, Student's *t*-test. **D:** Analysis of anti-ssDNA antibodies was performed by ELISA. Both sera from Tx+Ovx ($n = 5$) and Tx alone ($n = 5$) NFS/*sld* mice had negligible levels of IgG anti-ssDNA, whereas sera from MRL/*lpr* ($n = 6$) had high levels of antibodies. Results represent two experiments. * $P < 0.001$ from MRL/*lpr*, Mann-Whitney U test.

of estrogens, we found that the addition of estrogens clearly inhibited apoptosis of salivary gland cells *in vitro* (Figure 7B).

Discussion

It is believed that autoimmune diseases appear to be influenced by sex steroids in a non-uniform manner. In spontaneous autoimmune murine lupus, female mice display higher autoantibody titers, as well as more severe disease manifestation, than do male mice.⁴⁰ In NZB/NZW mice, testosterone suppresses both lupus development and autoantibody production⁴¹ and has been shown to improve survival.⁴² It is recently reported that testosterone therapy ameliorates experimental autoimmune encephalomyelitis in SJL/J mice.⁴³ In contrast, there are

experimental autoimmune conditions in which estrogens suppress disease developments such as adjuvant arthritis, experimental thyroiditis, and experimental autoimmune encephalomyelitis.^{44–46}

To analyze the *in vivo* role of estrogenic action on the development of autoimmune exocrinopathy, we investigated a murine model for SS in NFS/*sld* mutant mice treated with ovariectomy (Ovx). Histology of autoimmune lesions in estrogen-deficient SS model mice showed, to our surprise, severe destructive changes with inflammatory infiltration. Moreover, these lesions were dramatically prevented by exogenous estrogen treatment, but not by tamoxifen and testosterone treatment. These findings suggest that estrogenic action has an important protective role during development of autoimmune lesions in the murine SS model. It was reported that testosterone therapy leads to a dramatic suppression of the lymphocyte infiltration in animal models of SS, such as MRL/*lpr* and NZB/NZW1.^{47,48} This discrepancy could be explained that these SS-like lesions were accompanied by lupus-like systemic autoimmune disorders, whereas autoimmune lesions in our model are organ-specific ones in the salivary and lacrimal glands.

An estrogen deficiency induced by Ovx in the murine SS model results in a significant decrease in the splenic CD8⁺ T cell population *in vivo*, but a ConA-stimulated responsiveness of splenic CD8⁺ T cells with addition of estrogens was significantly higher than that of CD4⁺ T cells *in vitro*. As we detected an intense expression of estrogen receptor in splenic CD8⁺ T cells, it is possible that estrogen deficiency may induce disturbance of peripheral tolerance through CD8⁺ T cells bearing the suppressor phenotype. An immunoregulatory role for estrogens has been supported by numerous experimental and clinical observations, but the cellular targets of estrogens in the immune system have not been clearly defined. It was demonstrated that estrogens show selective binding for CD8⁺ T cells through estrogen receptors.⁴⁹ However, the opposite data are consistent with a role for CD4⁺ T cells as targets for estrogens.^{50,51} The present data of ours suggest a possible pathway for the immunomodulation of CD8⁺ T cells in the estrogen-deficient murine SS model.

We found a significant increase in serum autoantibody production against the organ-specific autoantigen 120-kd α -fodrin in estrogen-deficient SS model mice. It was reported that estrogens selectively reduce B cell precursors,⁵² and an estrogen deficiency stimulates B cell development in mouse bone marrow.⁵³ It was also shown that an estrogen deficiency stimulated autoantibody production,^{54–56} and an increase in autoantibody production by estrogen deficiency has been mediated by cytokines such as interleukin-6, IFN- γ , and tumor necrosis factor- α .^{57–60} Although we could not detect a prominent increase of peripheral B220⁺ or class-II⁺ cells (data not shown), a significant increase in LPS responsiveness of spleen cells was seen in the estrogen-deficient murine SS model. This is the first report to demonstrate that estrogen deficiency involves in both T and B cell responses on the development of organ-specific autoimmune lesions *in vivo*.

Figure 6. Detection of TUNEL⁺ apoptotic duct cells in the salivary gland sections from Tx+Ovx (A), Tx alone (B), and non-Tx NFS/*sld* mice (C). D: A significant increase of apoptotic epithelial duct cells was observed in the salivary gland tissues from Tx+Ovx NFS/*sld* mice in all ages. The percentage of duct cells staining positively with TUNEL was enumerated using a 10- × 20-grid net micrometer disk covering an objective of area 0.16 mm². Data were analyzed in 10 fields per section and were expressed as mean percentage ± SD in five mice examined per group. **P* < 0.01; ***P* < 0.001, Student's *t*-test.

The roles of the Fas/FasL system in the pathogenesis of autoimmune diseases have already been proposed.^{20–24} Fas-mediated apoptosis is recognized as a major pathway for the induction of the tissue damage in autoimmune diseases. It has been reported that both Fas and FasL are present in thyrocytes, and their concomitant expression on thyrocytes, independent of infiltrating T cells, is responsible for thyrocyte destruction in Hashimoto's thyroiditis.⁶¹ In contrast, expression of Fas by pan-

creatic β cells has been shown to have a major influence on the susceptibility of tissue destruction in nonobese diabetic (NOD) mice to diabetes.⁶² We demonstrated a significant increase of TUNEL⁺ apoptotic epithelial cells in the salivary glands in Tx plus Ovx mice at all ages. We also found that IFN- γ -induced Fas expression on these cells was reduced by the addition of estrogens. It was shown that a physiological concentration of estrogens augmented the activity of the IFN- γ promoter in mitogen-

Figure 7. *In vitro* effect of estrogens on Fas expression and apoptosis in the cultured salivary gland cells. **A:** These cells were analyzed for surface expression of Fas. Data are expressed as ratio of the mean fluorescence intensity (MFI) of Ab against the specific antigen relative to the MFI of control Abs for each treatment. MFI in IFN- γ -induced Fas expression was clearly reduced by estrogens, but not by tamoxifen. * $P < 0.01$, Student's *t*-test. **B:** These cells treated with IFN- γ and estrogens were additionally cultured for 24 hours in the presence of anti-Fas MAb and analyzed for apoptotic nuclei with PI. The addition of estrogens clearly blocked apoptosis of the cultured salivary gland cells. * $P < 0.01$; ** $P < 0.005$, Student's *t*-test.

stimulated murine spleen cells,⁶⁰ and the administration of exogenous estrogens could induce Fas-mediated apoptosis not only in cultured cells but also *in vivo*.⁶³ It was also demonstrated that 17 β -estradiol directly induces mammalian osteoclast apoptosis in a dose- and time-dependent manner.⁶⁴ In this study, we demonstrated experimental evidence that anti-Fas MAb-stimulated apoptosis in the salivary gland cells was clearly inhibited by the addition of estrogens.

In conclusion, we have demonstrated that endogenous estrogens may play a protective role on the development and progression of organ-specific autoimmune lesions in the murine SS model. Future therapeutic strategies for autoimmune diseases may be based on estrogen treatment designed to shift the autoantigen-specific T cell response as well as the B cell response.

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