

Figure 3. Subcellular distribution of intact and cleaved α -fodrin in human salivary cells. Ethanol-fixed human salivary HSG cells were stained with the human anti-150-kd α -fodrin Fabs SK415 (A) and SK460 (C), a human anti-HIV-1 gp120 Fab, b12 (E, negative control), and the mouse anti- α -fodrin mAb AA6 (I). Intense cytoplasmic staining with Fabs SK415 and SK460 and mouse mAb AA6 was observed in the permeabilized cells, although only partial co-localization between the human and mouse mAbs was observed. The cells were also stained with propidium iodide (B, D, F, H). Apoptotic HSG cells, exhibiting increased levels of cleaved 120- and 150-kd α -fodrin (J, 6H) compared to nonapoptotic cells (J, 0H), were also stained with Fab SK415 (G) and showed intense staining at the surface blebs.

Cleaved 150-kd α -Fodrin Is Present in Blebs of Apoptotic HSG Cells

Previous studies have shown that α -fodrin is cleaved by different apoptotic enzymes, but the question remained as to how α -fodrin, an intracellular protein, became ex-

posed to the immune system. One possibility is that 150-kd fragments of α -fodrin are exposed on the cell surface during the morphological and biochemical process of apoptosis. Recent reports have demonstrated that certain SS-associated autoantigens, including La, translocate during apoptosis to the cell surface, and particularly to cell surface blebs.³⁴⁻³⁶ To determine whether 150-kd α -fodrin fragments become concentrated at the surface of apoptotic HSG cells, apoptosis was induced in HSG cell cultures by tumor necrosis factor- α and cycloheximide, and the cells stained with the anti-150-kd α -fodrin Fab SK415 or anti-HIV-1 gp120 Fab (negative control), and propidium iodide (PI). Apoptotic cells were visualized by the binding of fluorescein isothiocyanate-annexin V to phosphatidylserine on the cell surface before fixation and by morphological appearance, including nuclear condensation, surface blebbing, and cell shrinkage (Figure 3, G and H). HSG cell cultures induced by tumor necrosis factor- α and cycloheximide to undergo apoptosis were also analyzed by Western blot and showed increased levels of cleaved 120- and 150-kd α -fodrin (Figure 3J, 6H) compared to noninduced HSG cell cultures, which contained only low amounts of constitutive apoptotic cells, as previously reported (Figure 3J, 0H).^{14,17} Confocal analysis demonstrated that anti-150-kd α -fodrin translocated to cell surface blebs of apoptotic HSG cells (Figure 3G). No staining was observed with a control human Fab Ab against HIV-1 gp120 (data not shown).

Localization of 150-kd Cleaved α -Fodrin within SS Salivary Gland Tissue

To determine the distribution of 150-kd fragments of α -fodrin in salivary tissue from patients with active SS, fresh-frozen labial biopsies from three SS patients were obtained and examined by immunohistochemistry. Cryostat tissue sections were stained with anti-150-kd α -fodrin Fab SK415 and a mouse anti- α -fodrin mAb AA6 (Figure 4, A and B). Laser-scanning confocal microscopy of the SS-infiltrated salivary gland using SK415 revealed intense staining of the acinar epithelia cells, with intensification at the cell surface and co-localization with the mouse anti- α -fodrin mAb (Figure 4C, solid arrows). However, SK415 also exhibited intense patchy staining corresponding to the lymphocytic infiltrate immediately surrounding the affected acini, an area not stained by the mAb AA6 or the fluorescein isothiocyanate-labeled anti-human F(ab')₂ Ab alone (Figure 4, open arrows). The SK415 staining in this area was not confined to cells, but seemed to be associated with 150-kd α -fodrin, which had leaked from inflamed glandular cells. The unaffected duct cells were stained by mAb AA6 (Figure 4, arrowheads), but only weakly by Fab SK415. Similarly, duct cells from normal salivary gland tissue were stained by mAb AA6, but not, or only weakly, by Fab SK415 (Figure 4D). The fixation method of the labial biopsy sections (ethanol or acetone) did not seem to influence the staining patterns of Fab SK415 and mAb AA6.

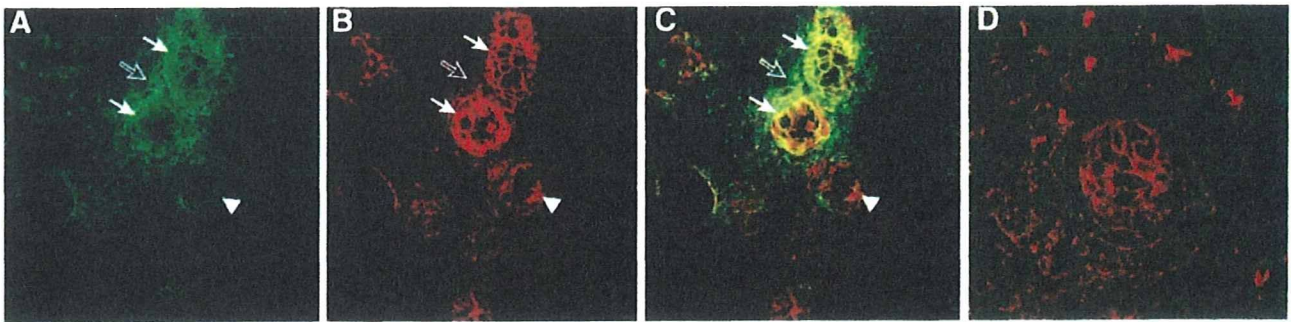


Figure 4. Distribution of cleaved and intact α -fodrin in salivary gland tissue of a SS patient with active disease. Tissue sections from salivary gland biopsies of patients with SS were stained with human anti-150-kd α -fodrin Fab SK415 (A, green) and mouse anti- α -fodrin mAb AA6 (B, red) and analyzed by laser-scanning confocal microscopy. Intense staining corresponding to acinar epithelia cells, and with intensification at the cell surface was observed with SK415 (solid arrows). Only partial co-localization was observed between Fab SK415 and mAb AA6 (C), because Fab SK415, but not mAb AA6, also stained areas corresponding to the lymphocytic infiltrate surrounding the affected acini (open arrow), whereas AA6, but not SK415, stained unaffected ducts (arrowhead). Duct cells from a normal salivary gland were stained with AA6, but not, or only weakly, with SK415 (D). No staining of the salivary gland tissue sections was observed with the negative control Fab b12 or a fluorescein isothiocyanate-labeled anti-human F(ab)₂ alone (not shown).

Discussion

Lymphocyte infiltration in the salivary glands leading to glandular destruction is a common finding in SS patients. The infiltrate consists of predominantly T cells as well as large numbers of B and plasma cells that actively produce immunoglobulin with autoimmune reactivity. To elucidate the components of this immunopathology, we examined the Ab response to α -fodrin in SS patients using serology and phage display cloning.

In the initial study proposing anti- α -fodrin Abs as a disease marker of SS, Western blot analysis of a large panel of Japanese SS patients showed that IgG Ab to 120-kd cleaved α -fodrin could be used as a marker for SS, because it exhibited high sensitivity (95%) and specificity (100%).⁷ Subsequently, anti-120-kd α -fodrin Abs were also found in some patients with SLE without SS.⁹ The high sensitivity and specificity of anti- α -fodrin Ab as a diagnostic marker of SS has been questioned,²¹ although other investigators have also found good correlation between SS and anti- α -fodrin Abs, although the sensitivity was lower than in the Japanese study.^{11,37} We investigated a panel of SS patients fulfilling the strict San Diego criteria by evaluating serum panels by both ELISA and Western blotting using purified mouse brain α -fodrin, similar to the antigen source used in the initial Japanese study. This antigen preparation contains both intact (240 kd) and cleaved forms (eg, 120 kd, 150 kd) of α -fodrin, and thus will also be recognized by Abs that specifically bind epitopes accessible only on the cleaved forms of α -fodrin, such as those cloned in this study. Our study demonstrated significant differences in sensitivity between ELISA and Western blotting, suggesting that a major part of the α -fodrin Ab response is directed against conformational epitopes, or that the affinity of the anti- α -fodrin Ab in some SS patients was not sufficient to allow detection by Western blotting. Nearly all (98%) of the SS patients were positive for α -fodrin by ELISA, whereas anti- α -fodrin Ab reactivity by Western blotting was significantly lower (52%), and only 36.0% stained the 120-kd α -fodrin fragment. Some differences in α -fodrin Ab profiles were observed between the American and the Japanese SS patient groups, probably because the two

groups represent two distinct populations with different genetic and environmental influences.

Interestingly, we found that ~25% of both the American and Japanese SS sera recognized a 150-kd fragment of cleaved α -fodrin, an Ab specificity not previously evaluated. This specificity was not seen in a panel of sera from healthy individuals and patients with SLE or RA. Molecular analysis of the anti-150-kd Ab response using IgG Ab phage display libraries generated from two of anti-150-kd seropositive SS patients yielded a panel of hmAbs that specifically recognized the 150-kd form of cleaved α -fodrin and not 120-kd or intact α -fodrin. The specificity of these hmAbs is distinct from that of the currently available mouse anti- α -fodrin mAbs recognizing 150-kd α -fodrin, which also bind 120-kd α -fodrin and intact α -fodrin.

In the *NFS/sld* SS mouse model, dysregulation of anti-fodrin CD4⁺ T cells leads to Fas-mediated apoptosis of salivary gland epithelial cells and a corresponding increase in clinical symptoms.⁸ In humans, a similar scenario has been proposed, and increased apoptosis has been observed in SS salivary glands. Fas expression in the salivary gland cells and FasL on the infiltrating CD4⁺ T cell in SS patients have been reported, although their roles in disease pathology remain to be elucidated.³⁸ In addition, CTL-mediated lysis/apoptosis through the granzyme and perforin pathways may be involved in salivary gland destruction.² In the mouse model,⁸ the increased apoptosis was accompanied by an increase in cleaved 120-kd α -fodrin in the affected tissue, as well as in serum anti-120-kd Ab levels, similar to our observation of cleaved 150-kd α -fodrin in SS patients. Indeed, treatment of mice with caspase inhibitors prevented induction of SS,¹⁹ suggesting that α -fodrin cleavage and anti- α -fodrin Ab production is more than an epiphenomenon. We found the 150-kd α -fodrin fragment within affected acinar epithelial cells, and also in the surrounding lymphocytic infiltrate. In the latter areas, the staining was not confined to cells, suggesting leakage of the 150-kd α -fodrin fragment from the apoptotic acinar/ductal epithelial cells into the surrounding interstitium. Interestingly, leakage of 150-kd fragments of α -fodrin has also been observed in tissue cultures of apoptotic human neuroblastoma

cells.³⁹ In contrast, the anti-150-kd Fabs stained normal salivary cells with lower intensity than a mouse mAb recognizing both intact and cleaved α -fodrin. Our observation seems to support earlier findings that low levels of 120-kd and 150-kd are present in normal cells, but are significantly increased during apoptosis.¹⁴ Our study also suggests that 150-kd α -fodrin is translocated to cell surface blebs in apoptotic HSG cells, similar to Ro/SSA,^{35,36} thereby possibly presenting it to the immune system and eliciting an Ab response. Previous studies have shown α -fodrin to be cleaved by calpain in apoptotic T cells, and by calpain and caspases in anti-Fas-stimulated Jurkat cells and/or neuronal apoptosis.¹⁵⁻¹⁷ In human SS, the 150-kd α -fodrin fragments are most likely the result of caspase cleavage,^{18,19} but this requires further analysis. In addition, α -fodrin can be cleaved by an unidentified protease present in granules of CD8⁺ CTLs.¹⁸ Interestingly, membrane blebbing during apoptosis has been suggested to be partially due to fodrin cleavage.¹⁴

In conclusion, our data supports the theory that in SS patients, α -fodrin is cleaved during apoptosis in the inflamed salivary gland tissue and elicits an Ab response to the cleaved products. Our study also showed that the Ab response to α -fodrin in SS patients is not limited to Abs against the 120-kd fragment, but includes other cleaved products, such as the 150-kd fragment. Inclusion of these Ab specificities would likely increase the sensitivity of the anti- α -fodrin Ab test, and enhance its usefulness as a diagnostic marker of human SS. The isolated human monoclonal Abs to cleaved α -fodrin may serve as useful reagents for diagnostic immunohistochemical analysis of SS salivary gland tissue and study of SS pathogenesis.

References

1. Fox RI, Tornwall J, Maruyama T, Stern M: Evolving concepts of diagnosis, pathogenesis, and therapy of Sjogren's syndrome. *Curr Opin Rheumatol* 1998, 10:446-456
2. Tapinos NI, Polihronis M, Tzioufas AG, Skopouli FN: Immunopathology of Sjogren's syndrome. *Ann Med Interne (Paris)* 1998, 149:17-24
3. Jonsson R, Moen K, Vestreim D, Szodoray P: Current issues in Sjogren's syndrome. *Oral Dis* 2002, 8:130-140
4. Fox R: Sjögren's Syndrome: Primer on the Rheumatic Diseases. Atlanta, Arthritis Foundation, 1998, pp 283-286
5. Vitali C, Bombardieri S, Moutsopoulos HM, Balestrieri G, Bencivelli W, Bernstein RM, Bjerrum KB, Braga S, Coll J, de Vita S, et al: Preliminary criteria for the classification of Sjogren's syndrome. Results of a prospective concerted action supported by the European Community. *Arthritis Rheum* 1993, 36:340-347
6. Vitali C, Bombardieri S, Moutsopoulos HM, Coll J, Gerli R, Hatron PY, Kater L, Kontinen YT, Manthorpe R, Meyer O, Mosca M, Ostuni P, Pellerito RA, Pennec Y, Porter SR, Richards A, Sauvezie B, Schiodt M, Sciuto M, Shoenfeld Y, Skopouli FN, Smolen JS, Soromenho F, Tishler M, Wattiaux MJ: Assessment of the European classification criteria for Sjogren's syndrome in a series of clinically defined cases: results of a prospective multicentre study. The European Study Group on Diagnostic Criteria for Sjogren's Syndrome. *Ann Rheum Dis* 1996, 55:116-121
7. Haneji N, Nakamura T, Takio K, Yanagi K, Higashiyama H, Saito I, Noji S, Sugino H, Hayashi Y: Identification of alpha-fodrin as a candidate autoantigen in primary Sjogren's syndrome. *Science* 1997, 276:604-607
8. Ishimaru N, Saegusa K, Yanagi K, Haneji N, Saito I, Hayashi Y: Estrogen deficiency accelerates autoimmune exocrinopathy in murine Sjogren's syndrome through Fas-mediated apoptosis. *Am J Pathol* 1999, 155:173-181
9. Watanabe T, Tsuchida T, Kanda N, Mori K, Hayashi Y, Tamaki K: Anti-alpha-fodrin antibodies in Sjogren syndrome and lupus erythematosus. *Arch Dermatol* 1999, 135:535-539
10. Miyagawa S, Yanagi K, Yoshioka A, Kidoguchi K, Shirai T, Hayashi Y: Neonatal lupus erythematosus: maternal IgG antibodies bind to a recombinant NH2-terminal fusion protein encoded by human alpha-fodrin cDNA. *J Invest Dermatol* 1998, 111:1189-1192
11. Witte T, Matthias T, Oppermann M, Helmke K, Peter HH, Schmidt RE, Tishler M: Prevalence of antibodies against alpha-fodrin in Sjogren's syndrome: comparison of 2 sets of classification criteria. *J Rheumatol* 2003, 30:2157-2159
12. Perrin D, Aunis D: Reorganization of alpha-fodrin induced by stimulation in secretory cells. *Nature* 1985, 315:589-592
13. Leto TL, Pleasic S, Forget BG, Benz Jr EJ, Marchesi VT: Characterization of the calmodulin-binding site of nonerythroid alpha-spectrin. Recombinant protein and model peptide studies. *J Biol Chem* 1989, 264:5826-5830
14. Martin SJ, O'Brien GA, Nishioka WK, McGahon AJ, Mahboubi A, Saido TC, Green DR: Proteolysis of fodrin (non-erythroid spectrin) during apoptosis. *J Biol Chem* 1995, 270:6425-6428
15. Martin SJ, Finucane DM, Amarante-Mendes GP, O'Brien GA, Green DR: Phosphatidylserine externalization during CD95-induced apoptosis of cells and cytoplasts requires ICE/CED-3 protease activity. *J Biol Chem* 1996, 271:28753-28756
16. Vanags DM, Porn-Ares MI, Coppola S, Burgess DH, Orrenius S: Protease involvement in fodrin cleavage and phosphatidylserine exposure in apoptosis. *J Biol Chem* 1996, 271:31075-31085
17. Nath R, Raser KJ, Stafford D, Hajimohammadreza I, Posner A, Allen H, Talanian RV, Yuen P, Gilbertsen RB, Wang KK: Non-erythroid alpha-spectrin breakdown by calpain and interleukin 1 beta-converting-enzyme-like protease(s) in apoptotic cells: contributory roles of both protease families in neuronal apoptosis. *Biochem J* 1996, 319:683-690
18. Nagaraju K, Cox A, Casciola-Rosen L, Rosen A: Novel fragments of the Sjogren's syndrome autoantigens alpha-fodrin and type 3 muscarinic acetylcholine receptor generated during cytotoxic lymphocyte granule-induced cell death. *Arthritis Rheum* 2001, 44:2376-2386
19. Saegusa K, Ishimaru N, Yanagi K, Mishima K, Arakaki R, Suda T, Saito I, Hayashi Y: Prevention and induction of autoimmune exocrinopathy is dependent on pathogenic autoantigen cleavage in murine Sjogren's syndrome. *J Immunol* 2002, 169:1050-1057
20. Maeno N, Takei S, Imanaka H, Oda H, Yanagi K, Hayashi Y, Miyata K: Anti-alpha-fodrin antibodies in Sjogren's syndrome in children. *J Rheumatol* 2001, 28:860-864
21. Witte T, Matthias T, Arnett FC, Peter HH, Hartung K, Sachse C, Wigand R, Braner A, Kalden JR, Lakomek HJ, Schmidt RE: IgA and IgG autoantibodies against alpha-fodrin as markers for Sjogren's syndrome. Systemic lupus erythematosus. *J Rheumatol* 2000, 27:2617-2620
22. Yanagi K, Ishimaru N, Haneji N, Saegusa K, Saito I, Hayashi Y: Anti-120-kDa alpha-fodrin immune response with Th1-cytokine profile in the NOD mouse model of Sjogren's syndrome. *Eur J Immunol* 1998, 28:3336-3345
23. Fox RI, Saito I: Criteria for diagnosis of Sjögren's syndrome. *Rheum Dis Clin North Am* 1994, 20:391-407
24. Cheney R, Levine J, Willard M: Purification of fodrin from mammalian brain. *Methods Enzymol* 1986, 134:42-54
25. Janicke RU, Ng P, Sprengart ML, Porter AG: Caspase-3 is required for alpha-fodrin cleavage but dispensable for cleavage of other death substrates in apoptosis. *J Biol Chem* 1998, 273:15540-15545
26. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987, 162:156-159
27. Persson MA, Caothien RH, Burton DR: Generation of diverse high-affinity human monoclonal antibodies by repertoire cloning. *Proc Natl Acad Sci USA* 1991, 88:2432-2436
28. Barbas III CF, Wagner J: Synthetic human antibodies selecting and evolving functional protein. *Methods CTMIE* 1995, 8:94-103
29. Burton DR, Barbas III CF, Persson MA, Koenig S, Chanock RM, Lerner RA: A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. *Proc Natl Acad Sci USA* 1991, 88:10134-10137

30. Maruyama T, Rodriguez LL, Jahrling PB, Sanchez A, Khan AS, Nichol ST, Peters CJ, Parren PW, Burton DR: Ebola virus can be effectively neutralized by antibody produced in natural human infection. *J Virol* 1999, 73:6024–6030
31. Warmerdam PA, van den Herik-Oudijk IE, Parren PW, Westerdal NA, van de Winkel JG, Capel PJ: Interaction of a human Fc gamma RIIB1 (CD32) isoform with murine and human IgG subclasses. *Int Immunol* 1993, 5:239–247
32. Ditzel HJ, Binley JM, Moore JP, Sodroski J, Sullivan N, Sawyer LS, Hendry RM, Yang WP, Barbas III CF, Burton DR: Neutralizing recombinant human antibodies to a conformational V2- and CD4-binding site-sensitive epitope of HIV-1 gp120 isolated by using an epitope-masking procedure. *J Immunol* 1995, 154:893–906
33. Binley JM, Ditzel HJ, Barbas III CF, Sullivan N, Sodroski J, Parren PW, Burton DR: Human antibody responses to HIV type 1 glycoprotein 41 cloned in phage display libraries suggest three major epitopes are recognized and give evidence for conserved antibody motifs in antigen binding. *AIDS Res Hum Retroviruses* 1996, 12:911–924
34. Casciola-Rosen LA, Anhalt G, Rosen A: Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *J Exp Med* 1994, 179:1317–1330
35. Baboonian C, Venables PJ, Booth J, Williams DG, Roffe LM, Maini RN: Virus infection induces redistribution and membrane localization of the nuclear antigen La (SS-B): a possible mechanism for autoimmunity. *Clin Exp Immunol* 1989, 78:454–459
36. Bachmann M, Chang S, Slor H, Kukules J, Muller WE: Shuttling of the autoantigen La between nucleus and cell surface after UV irradiation of human keratinocytes. *Exp Cell Res* 1990, 191:171–180
37. Zandbelt M, Degen W, van de Putte L, van Venrooij W, van den Hoogen Nijmegen F: Anti- α -fodrin antibodies: not specific nor sensitive for Sjögren's syndrome. *Arthritis Rheum* 1999, 42(Suppl):S142
38. Kong L, Ogawa N, Nakabayashi T, Liu GT, D'Souza E, McGuff HS, Guerrero D, Talal N, Dang H: Fas and Fas ligand expression in the salivary glands of patients with primary Sjögren's syndrome. *Arthritis Rheum* 1997, 40:87–97
39. Dutta S, Chiu YC, Probert AW, Wang KK: Selective release of calpain produced α -spectrin (α -fodrin) breakdown products by acute neuronal cell death. *Biol Chem* 2002, 383:785–791

Development of Autoimmune Exocrinopathy Resembling Sjögren's Syndrome in Estrogen-Deficient Mice of Healthy Background

Naozumi Ishimaru,* Rieko Arakaki,*
Megumi Watanabe,* Masaru Kobayashi,*†
Katsushi Miyazaki,*‡ and Yoshio Hayashi*

From the Department of Pathology,* Tokushima University School of Dentistry, and the Departments of Orthopedic Surgery,[†] and Otolaryngology,[‡] The University of Tokushima School of Medicine, Tokushima, Japan

Although a number of autoimmune diseases are known to develop in postmenopausal women, the mechanisms by which estrogen deficiency influences autoimmune lesions remain unclear. We speculate that antiestrogenic actions might be a potent factor in the formation of pathogenic autoantigens. Previously, we have identified 120-kd α -fodrin as an important autoantigen in Sjögren's syndrome (SS). When healthy C57BL/6 (B6) mice were treated with an ovariectomy (Ovx), we found a significant increase in TUNEL⁺-apoptotic epithelial cells in the salivary gland cells associated with α -fodrin cleavage during 2 and 3 weeks after Ovx. By contrast, no apoptotic cells were found in estrogen receptor- α knockout mice. In *in vitro* studies using primary cultured mouse salivary gland cells and human salivary gland cells, we found a cleavage product of 120-kd α -fodrin in cells that had undergone tamoxifen (Tam)-induced apoptosis through caspase activation, especially caspase-1. Adoptive transfer of α -fodrin-reactive T cells into Ovx-B6 and -SCID mice resulted in the development of autoimmune exocrinopathy quite similar to SS. These results suggest that estrogen deficiency exerts a crucial influence on autoantigen cleavage, and may cause, in part, autoimmune exocrinopathy in postmenopausal women. (*Am J Pathol* 2003, 163:1481-1490)

Loss of ovarian function following menopause results in functional failures of the immune system, bone metabolism, and endocrine system. Estrogenic action has been suggested to be responsible for the strong female preponderance of many autoimmune diseases, including systemic lupus erythematosus (SLE), scleroderma, rheumatoid arthritis (RA), and Sjögren's syndrome (SS).¹⁻⁴ Sex hormones influence both humoral and cell-mediated immune responses in a number of experimental models.⁵⁻⁹ Previous reports indicate that the increase in autoantibody production as a result of estrogen deficiency

is mediated by cytokines such as interleukin-6 (IL-6), interferon- γ (IFN- γ), and tumor necrosis factor- α (TNF- α), and that estrogen plays an important role in the regulation of B-lymphocyte development in mouse bone marrow.¹⁰⁻¹²

Recently, we have demonstrated that the dysfunction of regulatory T cells as a result of estrogen deficiency may play a crucial role on acceleration of organ-specific autoimmune lesions, and that estrogenic action influences target epithelial cells through Fas-mediated apoptosis in a murine model for SS.¹³ Although autoimmune diseases are triggered by various environmental factors, such as hormonal changes, microbial infections, stress, and aging,^{14,15} much less is known about the role of estrogen deficiency on the formation of autoantigen. We hypothesize that estrogen deficiency may influence the formation of pathogenic autoantigen in target organs through a T-cell-independent pathway.

Previously, we have identified 120-kd α -fodrin as an important autoantigen in both NFS/*s/d* murine SS model and in patients,¹⁶ but the mechanisms of α -fodrin cleavage in the salivary gland cells remain unclear. Our recent study has been strongly suggestive of essential roles of caspase cascade for α -fodrin cleavage leading to tissue destruction in primary SS.¹⁷ α -fodrin is a ubiquitous, heterodimeric calmodulin-binding protein that is cleaved by calcium-activated protease (calpain) in apoptotic cells and caspase through Fas-mediated apoptosis in Jurkat cells.¹⁸⁻²⁰ The fodrin α -subunit of various cells has also been shown to be cleaved in association with apoptosis.²¹⁻²³ Several reports have demonstrated that estrogen may play an inhibitory role on apoptosis in endothelial cells, breast cancer cells, cardiac myocytes, prostate cells, and neuronal cells.²⁴⁻²⁷ Moreover, it has been noted that some enzymatic activities are elevated in postmenopausal women compared with normal healthy women.^{28,29}

The aim of this study was to analyze the effect of estrogen deficiency on the formation of pathogenic autoantigen. Moreover, caspase activity in mouse salivary

This work was supported in part by Grants-in-Aid for Scientific Research (numbers 12307040 and 12557022) from the Ministry of Education, Science and Culture of Japan.

Accepted for publication June 26, 2003.

Address reprint requests to Yoshio Hayashi, Department of Pathology, Tokushima University School of Dentistry, 3 Kuramotocho, Tokushima 770, Japan. E-mail: hayashi@dent.tokushima-u.ac.jp.

gland cells stimulated by an antiestrogenic action has been analyzed, indicating that estrogen deficiencies may play a pivotal role in autoantigen cleavage initially triggered in the salivary and lacrimal gland.

Materials and Methods

Mice and Treatments

Female C57BL/6 (B6) mice (H-2^b) were purchased from Japan SLC (Shizuoka, Japan), and maintained in a specific pathogen-free (SPF) mouse colony and given food and water *ad libitum*. Estrogen receptor- α knockout mice (ER α KO) on a B6 strain background were purchased from Taconic (Germantown, NY). Female SCID C.B-17-scid/scid mice (H-2^d), purchased from Japan SLC (Shizuoka), were used to confirm cell transfer experiment. Normal female B6 (H-2^b), and BALB/c mice (H-2^d), purchased from Japan SLC (Shizuoka), were used to obtain antigen-stimulated T cells. Mice were ovariectomized (Ovx) at 4 weeks of age and compared with sham-operated (Sham, in both strain) mice. At 1 to 6 weeks after Ovx, all organs were removed from the mice and analyzed.

Histological Analysis

All organs were removed from mice, fixed with 4.0% phosphate-buffered formaldehyde (pH 7.2), and prepared for histological examination. The sections were stained with hematoxylin and eosin. The disease incidence was determined using the histological score of inflammatory lesions by White and Casarret,³⁰ estimated by three independent, well-trained pathologist in a blinded manner.

In Situ End-Labeling of Fragmented DNA (TUNEL)

Apoptotic cells were detected in sections using the *in situ* TUNEL Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Briefly, sections were incubated with proteinase K (20 μ g/ml) for 10 minutes, and then presoaked in TdT buffer (0.5 μ mol/L cacodylate, 1 μ mol/L CoCl₂, 0.5 μ mol/L dithiothreitol, 0.05% bovine serum albumin, 0.15 mol/L NaCl) for 10 minutes. Sections were incubated for 2 hours at 37°C in 25 ml of TdT solution, containing 1X terminal transferase buffer, 0.5 nmol of biotin-dUTP, and 10 U of TdT (Wako Pure Chemical). After the TdT reaction, sections were soaked in TdT blocking buffer (300 nmol/L NaCl, 30 mmol/L tri-sodium citrate-2-hydrate), incubated with HRP-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 DAB. Nuclei were counterstained with hematoxylin.

Western Blot Analysis

Western blot analysis with anti-human α -fodrin (Affiniti, Mamhead, UK) was performed. Briefly, the cells were

incubated in 20 mmol/L Tris-HCl (pH 8.0), 20 mmol/L NaCl, 0.5% Triton X-100, 5 mmol/L ethylenediaminetetraacetate (EDTA), and 3 mmol/L MgCl₂ lysis buffer. After centrifugation for 20 minutes at 12,000 \times g at 4°C, supernatant was extracted and used for sample. Ten micrograms of each sample per well was used for sodium dodecyl sulfide-polyacrylamide gel electrophoresis. Protein binding was visualized with enhanced chemiluminescence Western blotting reagent (Amersham Biosciences, Arlington Heights, IL). Control for protein loading was provided by anti-human α -tubulin, or GAPDH monoclonal antibody (Sigma Chemical Co., St. Louis, MO). To detect caspase-1 in cultured human cells and mouse tissues, Western blot analysis was performed by the indicated methods using anti-human caspase-1 (Sigma Chemical Co.), and anti-human active form caspase-1 (p20 subunit; Upstate Biotech, Charlottesville, VA) polyclonal antibody. An anti-human caspase-1 polyclonal antibody is known to cross-react with mouse lysate.

Primary Culture of Mouse Salivary Gland (MSG) Cells

Mouse salivary gland (MSG) epithelial cells were prepared as previously described.^{13,17} Briefly, mouse salivary glands were minced into 1-mm² pieces, washed with Hank's balanced salt solution (HBSS) without Ca²⁺ and Mg²⁺, and placed in a 60-mm dish 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. Fragments were washed with Dulbecco's modified Eagle's medium/soybean trypsin inhibitor, and placed in a mixture of collagenase (750 U/ml of type I) and hyaluronidase (500 U/ml of type IV) dissolved in DMEM/F12 containing 10% fetal bovine serum (FBS). The digest suspension was passed through a 100- μ m nylon mesh filter. Adherent cells after culture in DMEM containing 10% FBS for 24 hours at 37°C were isolated as salivary gland epithelial cells. Apoptotic cells were detected by flow cytometry with an EPICS flow cytometer (Beckman Coulter, Miami, FL) using the Annexin V-FITC Apoptosis Detection kit (Genzyme, Cambridge, MA).

Cell Culture

MSG cells, human salivary gland adenocarcinoma cell line (HSG),³¹ breast cancer cell line (MCF-7), colon cancer cells (HT29, Colo201), and Jurkat cells were cultured in the DMEM and RPMI-1640 including 10% FBS at 37°C. When the cells were treated with tamoxifen (Tam) (ICN Biochemicals, Costa Mesa, CA) and 17 β -estradiol (Wako Pure Chemical), they were cultured in the medium without FBS. Apoptotic cells were detected by flow cytometry with an EPICS flow cytometer (Beckman Coulter) using the Annexin V-FITC Apoptosis Detection kit (Genzyme).

Cell Transfection

We used polymerase chain reaction (PCR) techniques to generate derivatives of a human caspase-1 promoter-

luciferase construct. The following forward and reverse oligomers were used as primers to create a promoter of the human caspase-1 gene: 5'CACTGCAGATTGAGA-AACTCTTCACTG3', 5'GATCTAGAGGCTTTTCTCTCCT-CCCT3'. The PCR-amplified promoter fragments, including Pst-1/Xba-1 site, were cloned into the multiple cloning site of the pGL3-basic vector (Promega, Charbonnières, France), upstream of the luciferase gene. The caspase-1 promoter-luciferase gene were transfected into HSG cells using LipofectAMIN (Promega). The vector pGL3-basic (lacking a promoter) and the vector (Promega) pGL3-control served as negative and positive controls, respectively. Briefly, the transfection medium, containing 10 μ g of plasmid DNA and 60 μ l of Lipofectin reagent in 2 ml of serum-free DMEM was incubated for 20 minutes at room temperature and then diluted with serum-free DMEM to a final volume of 5 ml and added to HSG cells, plated the day before. The transfection process occurred at 37°C for 5 hours, then 5 ml of DMEM containing 20% fetal calf serum (FCS) was added to the cells.

Luciferase Assay

The transfected cells were incubated for 24 hours and stimulated during the last 2 hours with Tam (1×10^{-7} M). Pretreatment with 17 β -estradiol (1×10^{-9} M) was performed during last 12 hours. After rinsing with phosphate-buffered saline, cells were lysed with reporter lysis buffer (Promega), and cell extracts were used for luciferase assay with the Promega kit in a luminometer (Promega). To control transfection efficiency, pSV β -galactosidase plasmid (Promega) was cotransfected with the luciferase reporter constructs in a 1:4 ratio. The results showed that the difference in the relative efficiency of transfection between constructs was negligible.

Caspase Activities

Caspase activities in Tam-induced apoptosis in HSG cell extracts and mouse various tissues were assayed using Caspase-Family Colorimetric Substrate Set (BioVision Inc., Palo Alto, CA). Briefly, tissue or cell lysates were incubated with pNA-conjugated substrates (200 mmol/L, caspase-1, -2, -3, -5, -6, -8, and -9 substrate: Ac-YVAD-pNA, Ac-VDVAD-pNA, Ac-DEVD-pNA, Ac-WEHD-pNA, Ac-VEID-pNA, Ac-IETD-pNA, and Ac-LEHD-pNA) at 37°C for 2 hours. Absorbance of each sample was read at 405 nm in a microtiter plate reader using a 96-well plate. Fold-increase in caspase activity was determined by comparing these results with the level of the uninduced control. The caspase inhibitors z-VAD-fmk (1 μ mol/L), Ac-DEVD-CHO (1 μ mol/L), and Ac-WEHD-CHO (0.5 μ mol/L) (Sigma Chemical Co.) were added to the Tam-stimulated HSG cells. After the incubation for 48 hours, apoptotic cells were detected using Annexin-V flow cytometric analysis.

Adoptive Transfer

To obtain α -fodrin-reactive T cells, B6 mice were injected subcutaneously with 20 μ g recombinant α -fodrin protein

(JS-1) and Freund's complete adjuvant (ICN Biochemicals) at 4 weeks of age, and i.p. injections of 20 μ g JS-1 and Freund's incomplete adjuvant (ICN Biochemicals) were performed at 6 weeks of age. OVA (10 μ g/head)-reactive T cells were obtained in the same manner for control experiments. After 2 weeks later (8 weeks of age), mice were sacrificed and the splenic T cells were obtained as donor cells. As recipients, female B6 mice were ovariectomized (Ovx, $n = 7$) or sham-operated (Sham, $n = 5$) at 4 weeks of age. After 2 weeks (6 weeks of age), Ovx- and Sham-mice were transferred i.p. with 5×10^6 α -fodrin-reactive T cells. The transferred mice were analyzed at 4 and 8 weeks after the cell transfer. To confirm cell transfer experiments, Ovx- and Sham-SCID mice ($n = 7$ and $n = 5$) were transferred with α -fodrin-reactive T cells obtained from BALB/c mice in the same manner.

Proliferation Assay

Splenic T cells were performed on proliferation assay against various antigens including recombinant α -fodrin protein (JS-1). Single cell suspensions were cultured in 96-well flat bottom microtiter plate (5×10^5 cells/well) in RPMI-1640 containing 10% FCS, penicillin/streptomycin, and β -mercaptoethanol. Cells were cultured with 10 μ g/ml JS-1, and 2.0 μ g/ml Con A (EY Laboratories, San Mateo, CA). The control antigens used were OVA (5 μ g/ml), lysozyme (5 μ g/ml), and albumin (5 μ g/ml) (Sigma, St. Louis, MO). 3 [H]thymidine incorporation during the last 20 hours of the culture was evaluated using an automatoid β -liquid scintillation counter.

Results

Apoptosis Induced in Salivary Gland Cells by Ovariectomy (Ovx)

To examine the *in vivo* effects of estrogen deficiency in normal B6 and ER α KO mice, Ovx was performed at the age of 4 weeks. A radioimmunoassay confirmed that β -estradiol was not detectable in the sera of Ovx-mice (Ovx-B6 and Ovx-ER α KO, not detected; Sham-B6, 27.4 ± 2.8 pg/ml; Sham-ER α KO, 26.8 ± 3.6 pg/ml). At 1 to 6 weeks after Ovx, an *in situ* apoptosis detection assay was performed using all organs. Although we found a significant increase in TUNEL $^+$ apoptotic cells in the salivary gland sections of B6 mice, but not ER α KO mice, at 2 or 3 weeks after Ovx (Figure 1), no significant apoptosis was observed in any other organs of Ovx and Sham mice. To define TUNEL labeling due to the effect of Ovx *in vivo*, we examined Annexin V-flow cytometric analysis using primary cultured MSG cells, indicating that a small proportion of apoptotic cells (12.5%) in Ovx mice was found, but not in Sham mice (3.1%) (Figure 1A). No significant difference in number of TUNEL $^+$ apoptotic cells of Ovx and Sham mice was observed at 0, 1, 4, 5, and 6 weeks after Ovx (Figure 1B). Thus, apoptotic changes in the salivary glands of normal mice were observed transiently at 2 or 3 weeks after Ovx, supposing

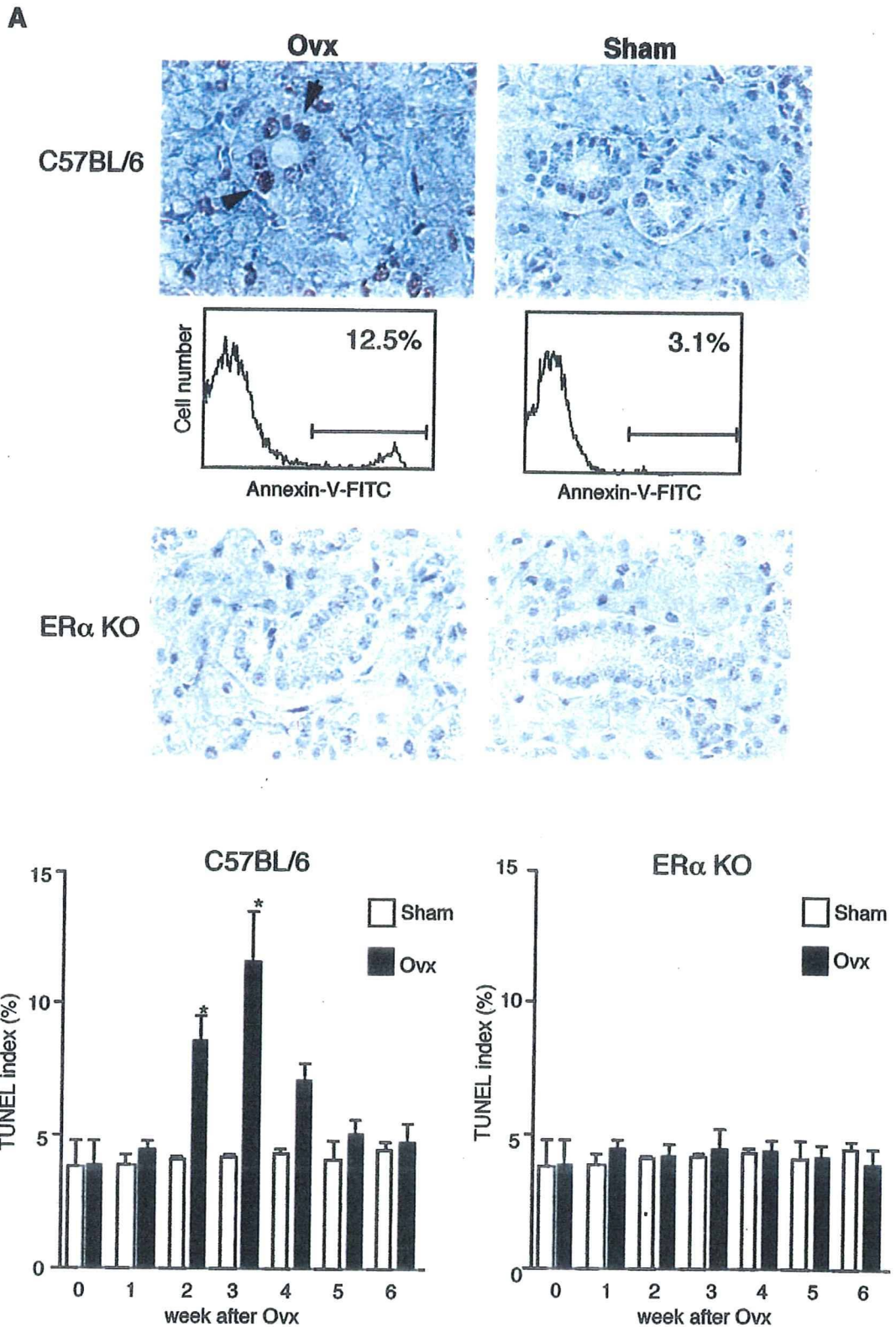


Figure 1. A: Detection of TUNEL⁺-apoptotic cells in the salivary gland sections from Ovx- and Sham-B6 mice, but not from ER α KO mice, at 3 weeks after Ovx (arrows). Annexin V-flow cytometric analysis using MSG cells demonstrated 12.5% positive cells detected in Ovx-B6 mice, but 3.1% positive in Sham-B6 mice. Data are representative in triplicate. **B:** A significant increase of apoptotic epithelial cells was observed in the salivary gland tissues from Ovx-B6 mice, not from ER α KO mice, restricted at 2 and 3 weeks after Ovx. The percentage of epithelial cells staining positively with TUNEL was enumerated using a 10- \times 20-grid net micrometer disk covering an objective of area 0.16 mm². Data were analyzed in 10 fields per section and expressed as mean percent \pm SD in five mice examined per group. (**P* < 0.05; Student's *t*-test.)

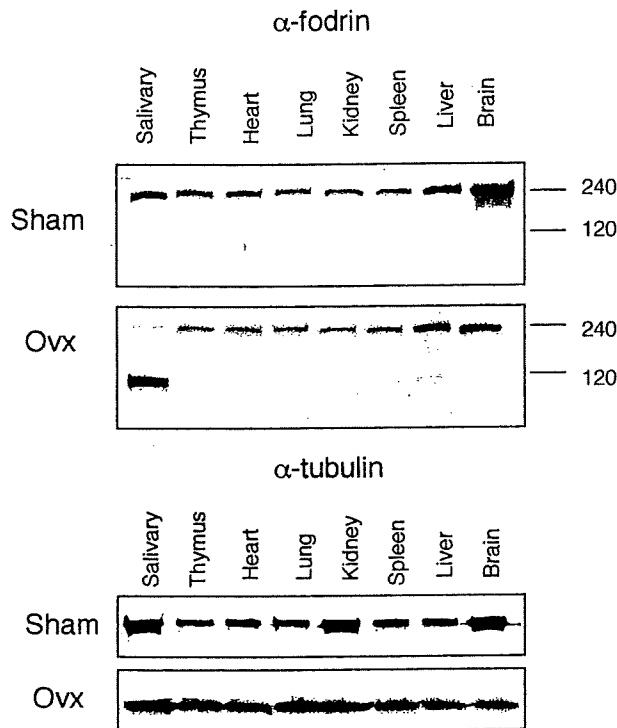


Figure 2. Detection of α -fodrin cleavage in various tissues from OvX- and Sham-B6 mice on Western blot analysis showing distinct band of 120 kd in the salivary gland tissue alone from OvX-B6 mice. α -tubulin protein as internal control was present. Data are representative of five mice in each group.

that antiestrogenic action to the epithelial tissues seems to be transient *in vivo*.

Effect of Estrogen Deficiency on α -Fodrin Proteolysis

We next investigated whether estrogen deficiency is involved in the formation of pathogenic autoantigens in salivary glands. To analyze α -fodrin proteolysis in the salivary glands from OvX-B6 mice, Western blot analysis was performed using tissue samples. Although an intense band expressing 120-kd α -fodrin was found in the salivary gland samples from OvX-B6 mice, no cleavage products of α -fodrin were observed in other organs from OvX mice or any of the samples from Sham mice, while expression of α -tubulin as a control was present (Figure 2). Thus, estrogen deficiency induces *in vivo* proteolysis of α -fodrin in association with apoptosis in the normal salivary gland cells.

Tamoxifen (Tam)-Induced Apoptosis and α -Fodrin Cleavage in Salivary Gland Cells

It has been reported that the antiestrogen tamoxifen (Tam) induces cell death in the human breast cancer cell line MCF-7.³² To examine whether Tam induces apoptosis in the mouse and human salivary gland (MSG and HSG) cells, the cells were treated with 1×10^{-10} to 1×10^{-6} (M) Tam for 48 hours. We found a time- and con-

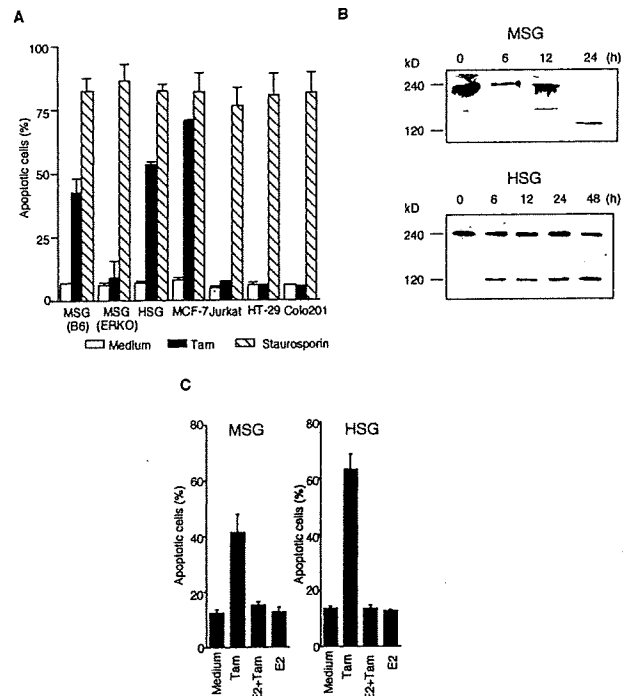


Figure 3. A: Effect of Tam (1×10^{-7} M) on apoptosis in various mouse and human cells (MSG, HSG, MCF-7, Jurkat, HT-29, and Colo201). Staurosporin ($1 \mu\text{mol/L}$) was used as common apoptotic reagent. B: α -fodrin cleavage into 120 kd in MSG and HSG cells induced by Tam. Western blot analysis with mouse monoclonal Ab to α -fodrin was performed in MSG and HSG cells stimulated with Tam (1×10^{-7} M) for 24 or 48 hours. The data are representative of three individual experiments. C: Tam-induced apoptosis of MSG and HSG cells was significantly reduced by the pretreatment with estrogen. Apoptotic cells were detected by flow cytometer using propidium iodide staining and Annexin V-FITC. The data are the mean \pm SD from three individual experiments.

centration-dependent increase in number of apoptotic MSG and HSG cells until 48 hours (data not shown). In contrast to the colon cancer cells (HT-29 and Colo201) or Jurkat cells, apoptosis was induced in MSG, HSG, and MCF7 cells treated with Tam (Figure 3A). Of importance is that the 240-kd α -fodrin in Tam-induced apoptotic MSG and HSG cells was cleaved into 120-kd fragment in a time-dependent manner on Western blotting (Figure 3B). In Tam-induced apoptotic MCF7 cells, negligible levels of cleaved products of α -fodrin were found on Western blotting. We next examined whether estrogen could inhibit Tam-induced apoptosis of MSG and HSG cells. As shown in Figure 3C, Tam-induced apoptosis of MSG and HSG cells was significantly reduced by the pretreatment with estrogen. These data indicate that apoptosis of mouse and human salivary gland cells followed by α -fodrin cleavage into 120-kd fragment could be induced by an antiestrogenic action.

Participation of Caspases in Estrogen-Deficient Salivary Gland Cells

We next investigated whether apoptotic proteases are involved in the α -fodrin cleavage during Tam-induced apoptosis in MSG and HSG cells. A significant increase in caspase-1 activity was detected with relatively elevated caspase-3 and -8 activity on Tam-induced apopto-

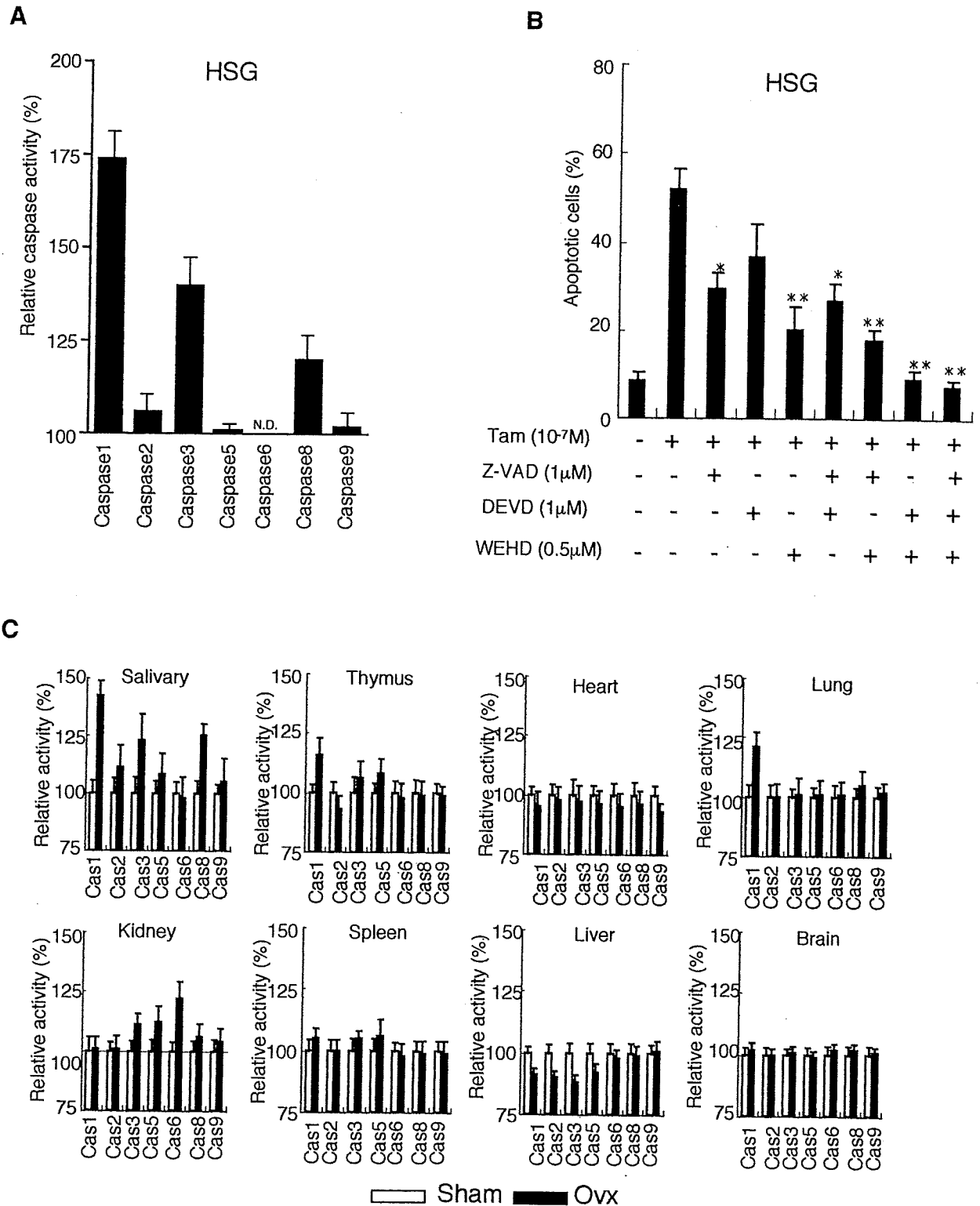


Figure 4. A: A significant increase in caspase-1 activity with relatively elevated caspase-3, and caspase-8 activity on Tam-induced apoptosis in HSG cells. B: Tam-induced apoptosis in HSG cells was inhibited considerably by the treatment with caspase inhibitors zVAD, DEVD, and WEHD. C: A significantly elevated caspase-1 activity in the salivary gland tissues is observed in Ovx-B6 mice with slight elevation of caspase-3, and caspase-8 activity. No significant differences in caspase activities were observed in other organs.

tic HSG cells (Figure 4A). In addition, Tam-induced apoptosis in HSG cells was inhibited considerably by treatment with caspase inhibitors zVAD, DEVD, and WEHD (Figure 4B). We next examined the level of the

caspase activities (caspase-1, -2, -3, -5, -6, -8, and -9) in various organs from Ovx- and non-Ovx-B6 mice. Figure 4C shows that a significantly elevated caspase-1 activity in the salivary gland tissues is observed in Ovx-B6 mice,

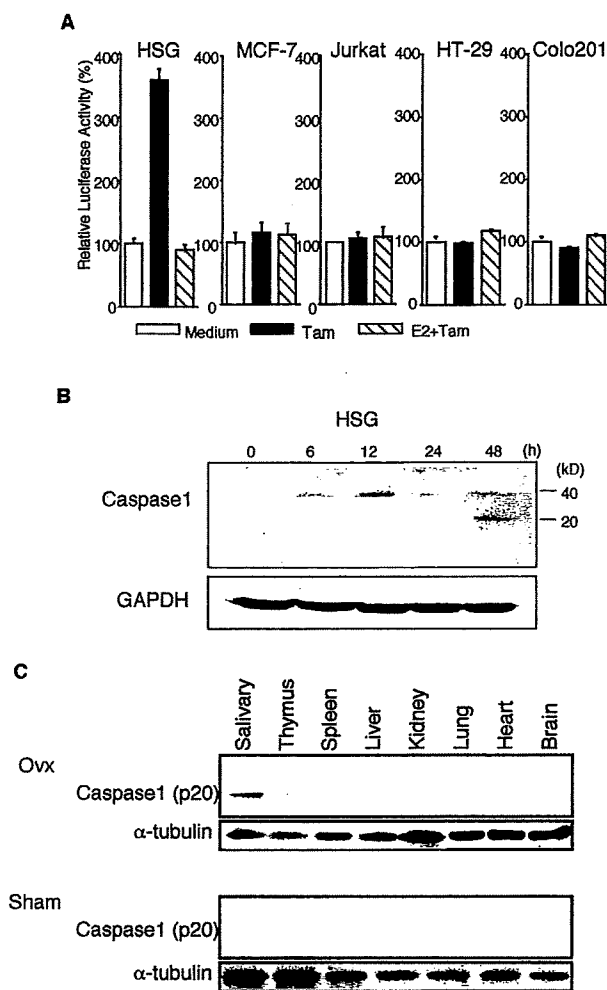


Figure 5. A: Luciferase assay of caspase-1 promoter activities in HSG cells stimulated with Tam. HSG, MCF-7, Jurkat, HT-29, and colo201 cells, after transfection with caspase-1 promoter Luc construct, were stimulated for 2 hours without or with 1×10^{-7} M Tam. Estrogen (17β -estradiol, 1×10^{-8} M) was added to the cells 12 hours before Tam stimulation. The results are the mean values of three independent experiments run in triplicate. **B:** Western blot analysis of caspase-1 in apoptotic HSG cells stimulated with Tam, showing an increase in procaspase-1 (40 kd) at 12 hours, and a time-dependent increase in caspase-1 active form (p20). Cytosolic extracts were prepared from HSG cells which were treated with Tam (1×10^{-7} M) for various times. **C:** Detection of caspase-1 active form (p20) in the salivary gland tissue, but not in various organs from Ovx- and Sham-B6 mice on Western blot analysis. α -tubulin proteins were used as internal control. Data were representative in triplicate.

with slight elevation of caspase-3 and 8 activity. No differences in caspase activities were observed in other organs. These results suggest that estrogen deficiency stimulates caspase activity, especially caspase-1, in the salivary gland tissues *in vivo*. We then examined whether Tam could influence the promoter activity of caspase-1 in HSG cells. After transfection with the plasmid containing the caspase-1 promoter-ligated upstream of the luciferase gene, HSG cells were stimulated with Tam, and then luciferase assay was performed. Figure 5A shows an increased promoter activity of caspase-1 after stimulation with Tam in HSG cells, but not in MCF-7, Jurkat, HT-29, and colo201 cells. The increase in caspase-1 promoter activity observed in HSG cells was significantly reduced by the addition of estrogen. We observed a

time-dependent expression of caspase-1 (p20) activities until 48 hours on Western blot analysis (Figure 5B). Figure 5C shows that a distinct expression of caspase-1 (p20) in the salivary gland tissues is observed in Ovx-B6 mice. These results suggest that the salivary gland cell apoptosis could be induced by caspase activation, especially caspase-1, in estrogen deficient state.

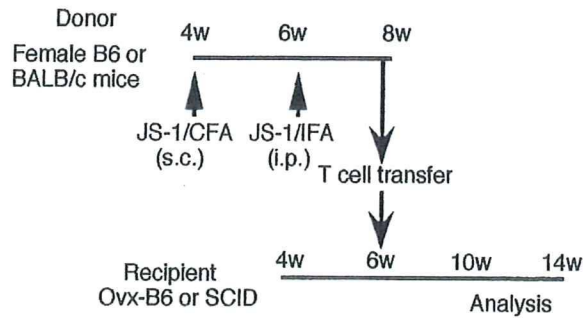
Adoptive Transfer of α -Fodrin-Reactive T Cells into Ovx-B6 and -SCID Mice

We examined the adoptive transfer experiments using α -fodrin-reactive T cells into Ovx-B6 mice as shown in protocol (Figure 6A). Before the cell transfer, we confirmed the proliferative T-cell response against JS-1, but not against lysozyme, albumin, and ovalbumin, of JS-1-immunized mice (Figure 6B). Consequently, inflammatory lesions developed exclusively in the salivary and lacrimal gland at 4 and 8 weeks after the transfer with 5×10^6 α -fodrin-reactive T cells, not with 5×10^6 OVA-reactive T cells, while no inflammatory lesions in any other organs were detectable (Table 1 and Figure 6C). When 5×10^6 α -fodrin-reactive CD4⁺ or CD8⁺ fractionated T cells were transferred into Ovx-B6 and Ovx-SCID mice, no inflammatory lesions were observed in any organs (data not shown). Proliferative T-cell response against recombinant α -fodrin (JS-1) was clearly observed in spleen cells from transferred Ovx-B6 mice (Figure 6D). In Ovx-SCID mice, inflammatory lesions were also induced in the salivary and lacrimal gland at 8 weeks after the transfer with 5×10^6 α -fodrin-reactive T cells, while no inflammatory lesions in any other organs were detectable (Table 1). Significant proliferative T-cell response against recombinant α -fodrin (JS-1) was observed in spleen cells from transferred Ovx-SCID mice (Figure 6E).

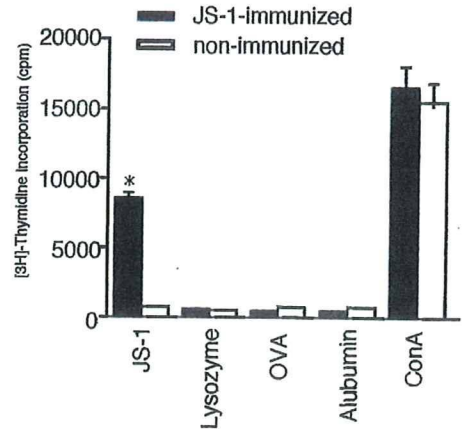
Discussion

The mechanisms responsible for the development of autoimmune diseases during the postmenopausal stage are still unclear. Previous reports concerning gender differences in autoimmunity have suggested that estrogen influences the cytokine production of effector cells and autoantibody production.^{33,34} The distinct immune environments in males and females underlie many of the gender-related differences in autoimmunity. These environments are established by the cytokines that are released by immune cells, particularly T helper (Th) lymphocytes. Sex hormones, pituitary hormones including prolactin, and growth hormones, as well as liver-derived insulin-like growth factor-1 affect autoimmune diseases by modulating cytokine productions.^{35,36} Women have higher levels of these hormones than men. Estrogen withdrawal after menopause leads to an increase in the production of cytokines, such as granulocyte-macrophage colony-stimulating factor, IL-1, IL-6, and TNF- α .³⁷ Although many studies have described the effects of estrogen on cytokine productions in effector cells, much less is known about the effect of estrogen deficiency in

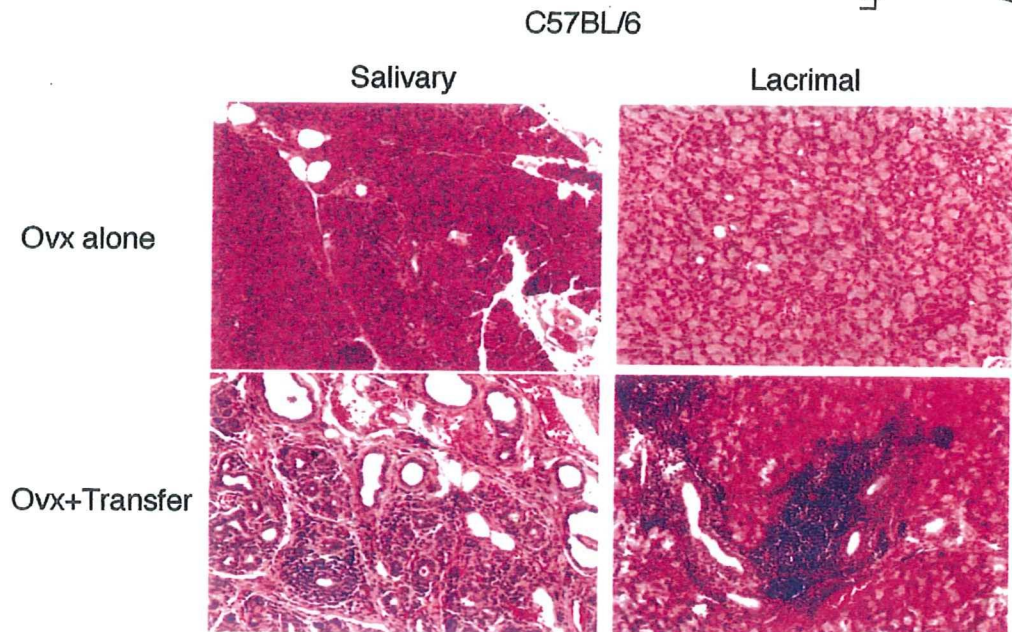
A



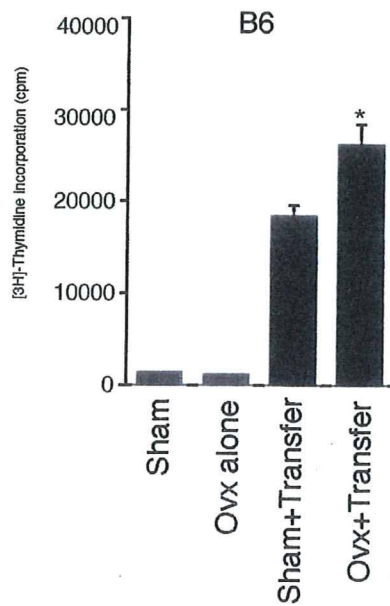
B



C



D



E

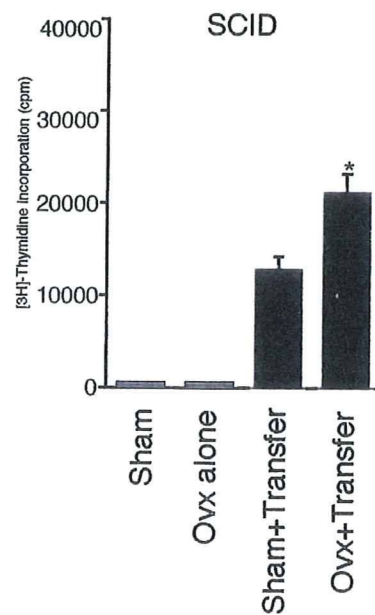


Table 1. Frequency of Inflammatory Lesions in the Salivary and Lacrimal Glands in Transferred C57BL/6 and SCID Mice Treated with Ovx

Treatment	No. of mice	No. of mice with lesions*		
		Submandibular	Parotid	Lacrimal
4 weeks after transfer (C57BL/6)				
Ovx alone [†]	5	0/5	0/5	0/5
Sham	5	0/5	0/5	0/5
Ovx+transfer [‡]	7	5/7	7/7	6/7
Sham+transfer	7	0/7	0/7	0/7
Ovx+transfer (OVA) [§]	5	0/5	0/5	0/5
8 weeks after transfer (C57BL/6)				
Ovx alone [†]	5	0/5	0/5	0/5
Sham	5	0/5	0/5	0/5
Ovx+transfer [§]	7	6/7	7/7	7/7
Sham+transfer	7	0/7	0/7	0/7
Ovx+transfer (OVA) [§]	5	0/5	0/5	0/5
8 weeks after transfer (SCID)				
Ovx alone [†]	5	0/5	0/5	0/5
Sham	5	0/5	0/5	0/5
Ovx+transfer [‡]	7	5/7	6/7	6/7
Sham+transfer	7	0/7	0/7	0/7

*Histological evaluation for frequency of inflammatory lesions in the salivary and lacrimal glands was done according to the method proposed by White and Casarett.³⁰

[†]Female C57BL/6 (B6) and SCID mice were ovariectomized (Ovx) at 4 weeks of age.

[‡]Ovx-B6 and SCID mice were transferred intraperitoneally with 5×10^6 α -fodrin-reactive T cells at 6 weeks of age.

[§]Ovx-B6 mice were transferred with 5×10^6 OVA-reactive T cells. α -fodrin-reactive T cells were obtained from B6 or BALB/c mice as described in detail in the text.

target organs of postmenopausal women. Thus, it is required to determine how estrogen deficiency influences the expression of autoantigens in target cells before the infiltration of lymphocytes into the salivary glands.

In this study, we have demonstrated a significant apoptosis associated with α -fodrin cleavage in the salivary gland cells of estrogen deficient healthy B6 mice. Moreover, inflammatory lesions developed exclusively in the salivary and lacrimal gland after the adoptive transfer with α -fodrin-reactive T cells in both Ovx-B6 and Ovx-SCID mice. These data indicate that α -fodrin cleavage triggered by estrogen deficiency plays a role in the development of autoimmune exocrinopathy in the salivary and lacrimal gland. By contrast, apoptotic cells in the salivary glands were not found in ER α KO mice. In *in vitro* studies using primary cultured MSG and HSG, we found a cleavage product of 120-kd α -fodrin in cells that had undergone Tam-induced apoptosis, not in other type of cells including MCF-7. Since pretreatment with estrogen inhibits the Tam-induced apoptosis of MSG and HSG cells, estrogen may play a crucial role in the apoptosis-related signal pathway. When we analyzed whether cysteine proteases are involved in Tam-induced apoptosis of HSG cells, we observed a time-dependent increase in the active forms of caspase-1. In addition, we found that the promoter activity of caspase-1 was significantly increased when HSG cells transfected with the promoter-caspase-1 gene were stimulated with Tam. Indeed, active forms of caspase-1 was detected only in the salivary gland tissues from Ovx-B6 mice. It has been reported

that estrogen inhibits the apoptosis of human myocytes and endothelial cells by reducing the activity of caspase-1-like protease.^{25,26} On the other hand, estrogen has been shown to inhibit bone resorption by directly inducing apoptosis of bone-resorbing osteoclasts.³⁸ Our results suggest that antiestrogenic actions might induce the salivary gland apoptosis through a caspase-1-mediated pathway. It is assumed that estrogen acts as a negative regulator of caspase-1 activity in the salivary gland cells.

When MSG and HSG cells were induced to undergo apoptosis using Tam, the 240-kd α -fodrin was cleaved into a single detectable fragment of 120 kd. Among the substrates cleaved during apoptosis are nuclear autoantigen targets for systemic autoimmune diseases such as PARP, U1-70-kd, the nuclear lamin, and DNA-dependent kinase.³⁹⁻⁴³ In organ-specific autoimmune diseases, no evidence in *in vivo* cleavage of self proteins during apoptosis has been demonstrated. Our data suggest that antiestrogenic actions have a potent effect on the proteolysis of α -fodrin autoantigen in the salivary gland through up-regulation of caspase-1 activity. These results strongly suggest that α -fodrin fragments induced by Ovx may play an important role on the development of autoimmune lesions as a pathogenic autoantigen. Molecular mechanisms responsible for tissue-specific apoptosis induced by estrogen deficiency are further investigated.

In conclusion, we have demonstrated that antiestrogenic actions, including estrogen deficiency or Tam stimulation, may have a crucial influence on apoptosis and

Figure 6. A: Experimental protocol of adoptive transfer using B6 and SCID mice as indicated. B: Significant proliferative response of the splenic T cells against recombinant α -fodrin (JS-1, 10 μ g/ml) were confirmed before the transfer (* $P < 0.01$, Student's *t*-test). Antigen-stimulated blastogenesis was measured in spleen T cells from immunized and non-immunized mice. No proliferative responses were found to lysozyme (5 μ g/ml), albumin (5 μ g/ml), and OVA (5 μ g/ml). Data are expressed as counts per minute \pm SD in triplicate. C: Representative histological features of the salivary and lacrimal glands in Ovx+transfer, and Ovx-alone mice (hematoxylin and eosin, magnification, $\times 120$). D: Significant proliferative response of the splenic T cells against recombinant α -fodrin (JS-1, 10 μ g/ml) in Ovx+transfer B6 mice was observed (* $P < 0.01$, Student's *t*-test). E: Significant proliferative response of the splenic T cells against recombinant α -fodrin (JS-1, 10 μ g/ml) in Ovx+transfer SCID mice was observed (* $P < 0.05$, Student's *t*-test). Data are expressed as counts per minute \pm SD in triplicate.

α -fodrin proteolysis through an increased caspase activity in the salivary gland cells, suggesting a novel mechanism for the development of organ-specific autoimmunity in postmenopausal women.

References

1. Lahita RG: The role of sex hormones in systemic lupus erythematosus. *Curr Opin Rheumatol* 1999, 11:352-356
2. Bateman A, Singh A, Kral T, Solomon S: The immune-hypothalamic-pituitary-adrenal axis. *Endocrinol Rev* 1989, 10:92-112
3. Grossman C: Possible underlying mechanisms of sexual dimorphism in the immune response, fact and hypothesis. *J Steroid Biochem* 1989, 34:241-251
4. Mooradian A, Morley J, Korenman S: Biological actions of androgens. *Endocrinol Rev* 1987, 8:1-28
5. Keisler LW, Vom-Saal FS, Keisler DH, Walker SE: Hormonal manipulation of the prenatal environment alters reproductive morphology and increases longevity in autoimmune NZB/W mice. *Biol Reprod* 1991, 44:707-716
6. Carlsten H, Nilsson N, Jonsson R, Backman K, Holmdahl R, Tarkowski A: Estrogen accelerates immune complex glomerulonephritis but ameliorates T cell-mediated vasculitis and sialadenitis in autoimmune MRL lpr/lpr mice. *Cell Immunol* 1992, 144:190-202
7. Jansson L, Olsson T, Holmdahl R: Estrogen induces a potent suppression of experimental autoimmune encephalomyelitis and collagen-induced arthritis in mice. *J Neuroimmunol* 1994, 53:203-207
8. Hawkins T, Gala R, Dunbar J: The effect of neonatal sex hormone manipulation on the incidence of diabetes in nonobese diabetic mice. *Proc Soc Exp Biol Med* 1993, 202:201-205
9. Jansson L, Mattsson A, Mattsson R, Holmdahl IR: Estrogen-induced suppression of collagen arthritis: V: physiological level of estrogen in DBA/1 mice is therapeutic on established arthritis, suppresses anti-type II collagen T-cell dependent immunity and stimulates polyclonal B-cell activity. *J Autoimmun* 1990, 3:257-270
10. Ishimi Y, Miyaura C, Ohmura M, Onoe Y, Sato T, Uchiyama Y, Ito M, Wang X, Suda T, Ikegami S: Selective effects of genistein, a soybean isoflavone, on B-lymphopoiesis and bone loss caused by estrogen deficiency. *Endocrinol* 1999, 140:1893-1900
11. Masuzawa T, Miyaura C, Onoe Y, Kusano K, Ohta H, Nozawa S, Suda T: Estrogen deficiency stimulates B lymphopoiesis in mouse bone marrow. *J Clin Invest* 1994, 94:1090-1097
12. Poli V, Balena R, Fattori E, Markatos A, Yamamoto M, Tanaka H, Ciliberto G, Rodan GA, Costantini F: Interleukin-6 deficient mice are protected from bone loss caused by estrogen depletion. *EMBO J* 1994, 13:1189-1196
13. Ishimaru N, Saegusa K, Yanagi K, Haneji N, Saito I, Hayashi Y: Estrogen deficiency accelerates autoimmune exocrinopathy in murine Sjogren's syndrome through Fas-mediated apoptosis. *Am J Pathol* 1999, 155:173-181
14. Mayes M: Epidemiologic studies of environmental agents and systemic autoimmune diseases. *Environ Health Perspect* 1999, 107:743-748
15. Zhao Z, Granucci F, Yeh L, Schaffer P, Cantor H: Molecular mimicry by herpes simplex virus-type 1: autoimmune disease after viral infection. *Science* 1998, 279:1344-1347
16. Haneji N, Nakamura T, Takio K, Yanagi K, Higashiyama H, Saito I, Noji S, Sugino H, Hayashi Y: Identification of α -fodrin as a candidate autoantigen in primary Sjogren's syndrome. *Science* 1997, 276:604-607
17. Saegusa K, Ishimaru N, Yanagi K, Mishima K, Arakaki R, Suda T, Saito I, Hayashi Y: Prevention and induction of autoimmune exocrinopathy is dependent on pathogenic autoantigen cleavage in murine Sjogren's syndrome. *J Immunol* 2002, 169:1050-1057
18. Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, Yuan J: Human ICE/CED-3 protease nomenclature (letter). *Cell* 1996, 87:171
19. Martin SJ, O'Brien GA, Nishioka WK, McGahon AJ, Mahboubi A, Saido TC, Green DR: Proteolysis of fodrin (non-erythroid spectrin) during apoptosis. *J Biol Chem* 1995, 270:6425-6428
20. Martin SJ, Finucane DM, Amarante-Mendes GP, O'Brien GA, Green DR: Phosphatidylserine externalization during CD95-induced apoptosis of cells and cytoplasts requires ICE/CED-3 protease activity. *J Biol Chem* 1996, 271:28753-28756
21. Janicke RU, Sprengart ML, Wati MR, Porter AG: Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *J Biol Chem* 1998, 273:9357-9360
22. Cryns VL, Bergeron L, Zhu H, Li H, Yuan J: Specific cleavage of α -fodrin during Fas- and tumor necrosis factor-induced apoptosis is mediated by an interleukin-1 β -converting enzyme/Ced-3 protease distinct from the poly(ADP-ribose) polymerase protease. *J Biol Chem* 1996, 271:31277-31282
23. Wang KK, Posmantur R, Nath R, McGinnis K, Whitton M, Talanian RV, Glantz SB, Morrow JS: Simultaneous degradation of α II- and β II-spectrin by caspase-3 (CPP32) in apoptotic cells. *J Biol Chem* 1998, 273:22490-22497
24. Szende B, Romics I, Vass L: Apoptosis in prostate cancer after hormonal treatment (letter). *Lancet* 1993, 342:1422
25. Spyridopoulos I, Sullivan A, Kearney M, Isner J, Losordo D: Estrogen-receptor-mediated inhibition of human endothelial cell apoptosis: estradiol as a survival factor. *Circulation* 1997, 95:1505-1514
26. Pelzer T, Schumann M, Neumann M, DeJager T, Stimpel M, Serfling E, Neyses L: 17 β -estradiol prevents programmed cell death in cardiac myocytes. *Biochem Biophys Res Commun* 2000, 268:192-200
27. Pike CJ: Estrogen modulates neuronal Bcl-xL expression and β -amyloid-induced apoptosis: relevance to Alzheimer's disease. *J Neurochem* 1999, 72:1552-1563
28. Acarturk F, Robinson JR: Vaginal permeability and enzymatic activity studies in normal and ovariectomized rabbits. *Pharm Res* 1996, 13:779-783
29. Oner P, Bekpınar S, Cinar F, Argun A: Relationship of some endogenous sex steroid hormones to leukocyte arylsulphatase A activities in pre- and postmenopausal healthy women. *Horm Metab Res* 1994, 26:301-304
30. White SC, Casarett GW: Induction of experimental autoallergic sialadenitis. *J Immunol* 1974, 112:178-185
31. Shirasuna K, Sato M, Miyazaki T: A neoplastic epithelial duct cell line established from an irradiated human salivary gland. *Cancer* 1981, 48:745-752
32. Kyprianou N, English HF, Davidson NE, Isaacs JT: Programmed cell death during regression of the MCF-7 human breast cancer following estrogen ablation. *Cancer Res* 1991, 51:162-166
33. Ansar-Ahmed S, Penhale WJ, Talal N: Sex hormones, immune responses, and autoimmune diseases. Mechanisms of sex hormone action. *Am J Pathol* 1985, 121:531-551
34. Ansar-Ahmed S, Dauphinee M, Montoya A, Talal N: Estrogen induces normal murine CD5⁺ B cells to produce autoantibodies. *J Immunol* 1989, 142:2647-2653
35. Luca IC: Prolactin secretion and the immune system. *Rev Med Chir Soc Med Nat Iasi* 1997, 101:56-59
36. Whitacre CC, Reingold SC, O'Looney PA: A gender gap in autoimmunity. *Science* 1999, 283:1277-1278
37. Kimble RB, Srivastava S, Ross FP, Matayoshi A, Pacifici R: Estrogen deficiency increases the ability of stromal cells to support murine osteoclastogenesis via an interleukin-1 and tumor necrosis factor-mediated stimulation of macrophage colony-stimulating factor production. *J Biol Chem* 1996, 271:28890-28897
38. Kameda T, Mano H, Yuasa T, Mori Y, Miyazawa K, Shiokawa M, Nakamaru Y, Hiroi E, Hiura K, Kameda A, Yang N, Hakeda Y, Kumegawa M: Estrogen inhibits bone resorption by directly inducing apoptosis of the bone-resorbing osteoclasts. *J Exp Med* 1997, 186:489-495
39. Casciola-Rosen L, Nicholson DW, Chong T, Rowan KR, Thornberry NA, Miller DK, Rosen A: Apoptain/CPP32 cleaves proteins that are essential for cellular repair: a fundamental principle of apoptotic death. *J Exp Med* 1996, 183:1957-1964
40. Oberhammer FA, Hochegger K, Froschl G, Tiefenbacher R, Pavelka M: Chromatin condensation during apoptosis is accompanied by degradation of lamin A+B, without enhanced activation of cdc2 kinase. *J Cell Biol* 1994, 126:827-837
41. Casciola-Rosen LA, Anhalt GA, Rosen A: DNA-dependent protein kinase is one of a subset of autoantigens specifically cleaved early during apoptosis. *J Exp Med* 1995, 182:1625-1634
42. Tu S, Cerione RA: Cdc42 is a substrate for caspases and influences Fas-induced apoptosis. *J Biol Chem* 2001, 276:19656-19663
43. Slee EA, Adrain C, Martin SJ: Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *J Biol Chem* 2001, 276:7320-7326

Development of Autoimmune Exocrinopathy Resembling Sjögren's Syndrome in Adoptively Transferred Mice With Autoreactive CD4+ T Cells

Rieko Arakaki,¹ Naozumi Ishimaru,¹ Ichiro Saito,¹ Masaru Kobayashi,² Natsuo Yasui,² Takayuki Sumida,³ and Yoshio Hayashi¹

Objective. The pathologic mechanisms responsible for organ-specific tissue damage in primary Sjögren's syndrome (SS) remain unclear, but it has been suggested that the pathology is mediated by autoreactive CD4+ T cells infiltrating the salivary and lacrimal glands. This study was undertaken to investigate whether α -fodrin autoantigen-specific autoreactive CD4+ T cells are capable of inducing autoimmune lesions.

Methods. A total of 45 synthetic α -fodrin peptides designed to be 20 amino acid residues in length were generated. To establish an autoreactive T cell line, limiting dilution analysis (LDA) was performed on lymph node cells (LNCs) in the presence of α -fodrin peptides. The effects of adoptive transfer of autoreactive CD4+ T cells into normal syngeneic recipients were investigated.

Results. Autoreactive CD4+ T cell lines that recognize synthetic α -fodrin peptide, which produced Th1 cytokines and showed cytotoxic activities, were established in a murine model for SS. T cell receptor V β usage and third complementarity-determining region (CDR3) sequences indicated that in some cases V β 6-CDR3 genes matched between the tissue-infiltrating T cells and the autoreactive T cell lines. Adoptive transfer

of the autoreactive CD4+ T cells into normal syngeneic recipients induced autoimmune lesions quite similar to those of SS.

Conclusion. Our data help to elucidate the pathogenic mechanisms responsible for tissue destruction in autoimmune exocrinopathy and indicate that autoreactive CD4+ T cells play a pivotal role in the development of murine SS.

Primary Sjögren's syndrome (SS) is a T cell-mediated autoimmune disease, and autoreactive T cells bearing the CD4 molecule may recognize unknown self antigen, triggering autoimmunity in the salivary and lacrimal glands and leading to clinical symptoms of dryness of the mouth and eyes (sicca syndrome) (1,2). Previously, we identified involvement of a 120-kd α -fodrin autoantigen in the pathogenesis of primary SS in humans and rodents (3,4), but the mechanisms for tissue destruction in target organs remain unclear.

Although an important role for T cells in the development of organ-specific autoimmune disease has been suggested, it is not known whether disease is initiated by a restricted inflammatory reaction to an organ-specific autoantigen. In most cases, antigenic challenge results in the establishment of immunologic memory, a state in which the immune system is maintained to respond effectively upon recurrent antigenic exposure. Autoreactive T cells generally respond to a limited number of immunodominant epitopes in self antigenic proteins, including myelin basic protein, thyroglobulin, and glutamic acid decarboxylase (5–8). Thymectomy on day 3 after birth (3d-Tx) is followed by the development of organ-specific autoimmune diseases (9,10). The *sld* mutation in NFS/N mice (NFS/*sld*, H-2D^a) is involved in the mucous cell differentiation of the sublingual gland (11). Using 3d-Tx NFS/*sld* mutant

Supported in part by Grants-in-Aid for Scientific Research (nos. 12307040 and 12557022) from the Ministry of Education, Science and Culture of Japan.

¹Rieko Arakaki, PhD, Naozumi Ishimaru, DDS, PhD, Ichiro Saito, DDS, PhD, Yoshio Hayashi, DDS, PhD: Tokushima University School of Dentistry, Tokushima, Japan; ²Masaru Kobayashi, MD, Natsuo Yasui, MD: University of Tokushima, Tokushima, Japan; ³Takayuki Sumida, MD: University of Tsukuba, Ibaraki, Japan.

Address correspondence and reprint requests to Yoshio Hayashi, DDS, PhD, Department of Pathology, Tokushima University School of Dentistry, 3 Kuramotocho, Tokushima 770, Japan. E-mail: hayashi@dent.tokushima-u.ac.jp.

Submitted for publication November 11, 2002; accepted in revised form August 20, 2003.

mice, we have established and analyzed a murine model of primary SS in which the animals spontaneously develop a disease with many of the characteristics of human SS (12–14). The T cell receptor (TCR) $V_{\beta}8$ and $V_{\beta}6$ genes are preferentially used in these lesions from the onset of disease in the 3d-Tx NFS/*sld* mouse model (12). However, little is known about the events triggering T cell invasion of the the salivary and lacrimal glands in prelude to the development of autoimmune lesions.

Alpha-fodrin is an actin-binding protein that is found at the periphery of chromaffin cells and may be involved in secretion (15). The stimulation of secretion in parotid acinar cells is associated with dramatic rearrangements of the sub-plasmalemmal cytoskeleton of α -fodrin (16). In the present study we established α -fodrin-reactive T cell lines capable of inducing autoimmune lesions similar to those found in SS. Moreover, the TCR V_{β} usage and the third complementarity-determining region (CDR3) sequences of the autoreactive T cell lines were determined.

MATERIALS AND METHODS

Mice. NFS/N mice carrying the mutant gene *sld* (11) were raised in our specific pathogen-free mouse colony. Thymectomy was performed on day 3 after birth.

Histologic and immunohistologic analysis. All organs were removed from the mice, and the sections were stained with hematoxylin and eosin. Histologic grading of the inflammatory lesions was done determined as described previously (17). Immunohistologic analysis was performed by the avidin-biotin-immunoperoxidase method utilizing ABC reagent (Vector, Burlingame, CA). Monoclonal antibodies used were as follows: biotinylated rat monoclonal antibodies to CD4 and CD8 (Cedarlane, Hornby, Ontario, Canada), Mac-1 (Becton Dickinson, Burlingame, CA), and B220, $V_{\beta}8$, $V_{\beta}6$, interleukin-2 (IL-2), IL-4, and interferon- γ (IFN γ) (all from PharMingen, San Diego, CA).

Recombinant α -fodrin autoantigen. Recombinant α -fodrin protein, the complementary DNA (cDNA) encoding human α -fodrin (JS-1: 1–1784 bp, 2.7A: 2258–4884 bp, 3'DA: 3963–7083 bp) (18) was constructed by inserting cDNA into the *Eco* RI site of pGEX-4T 1, 2, and 3. The mouse sequences of α -fodrin are identical to the human sequences.

Assessment of proliferative T cell response. Single cell suspensions of spleen cells and lymph node cells (LNCs) were cultured in 96-well flat-bottomed microtiter plates (5×10^5 cells/well) in RPMI 1640 containing 10% fetal calf serum (FCS), penicillin/streptomycin, and β -mercaptoethanol. Cells were cultured with each recombinant α -fodrin protein (JS-1, 2.7A, and 3'DA) ($5 \mu\text{g/ml}$). During the last 8 hours of the 72-hour culture period, $1 \mu\text{Ci}$ of ^3H -thymidine was added per well, and the incorporated radioactivity was determined using an automated beta liquid scintillation counter. We isolated tissue-infiltrating mononuclear cells from affected salivary

glands as described previously (14). Infiltrating T cells were purified using nylon wool (Wako, Osaka, Japan).

TCR V_{β} usage and CDR3 sequencing of polymerase chain reaction (PCR) products. To investigate the comparison of clonotypes of infiltrating T cells in vivo and autoreactive T cell lines, reverse transcriptase PCR (RT-PCR) was used to discriminate the diversities in the D, J, and N regions. Total RNA was prepared with Isogen (Nippon Gene, Tokyo, Japan), and amplification was performed with *Taq* polymerase with 5' primer specific for the TCR $V_{\beta}6$ and $V_{\beta}8$ genes and a 3' primer specific for the TCR C_{β} gene. The diluted sample ($2 \mu\text{l}$) was electrophoresed in nondenaturing 5% polyacrylamide gels containing 10% glycerol. After electrophoresis, the DNA was transferred to Immobilon-S (Millipore, Intertech, Bedford, MA) and hybridized with biotinylated C_{β} probe, streptavidin, biotinylated alkaline phosphatase, and a chemiluminescent substrate system (Millipore Intertech). The inserted TCR genes were sequenced with dye-labeled primers and AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), using a 373A automated DNA sequencing system (Applied Biosystems).

Synthetic peptides. Peptides were synthesized using TBOC chemistry with a model 430A peptide synthesizer (Applied Biosystems, CA). A total of 45 synthetic peptides designed to be 20 amino acid residues in length, overlapping by 5 amino acid residues, were generated.

Autoreactive T cell line. To establish an α -fodrin peptide-specific T cell line, limiting dilution analysis (LDA) was performed as described previously (19,20), on LNCs in the presence of α -fodrin peptides and irradiated syngeneic spleen cells. LNCs from 3d-Tx NFS/*sld* mice (5 weeks old) were cultured with α -fodrin peptides ($10 \mu\text{g/ml}$) in RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, and 100 units ($100 \mu\text{g/ml}$) 100 penicillin/streptomycin in 96-well plates at 1×10^6 cells/well. On day 3, IL-2 (Genzyme, Cambridge, MA) was added; cells were fed with media containing 0.5 ng/ml IL-2 every 3 days. On day 14, an aliquot was analyzed for reactivity to α -fodrin peptides. Cells (1×10^4) from each cell line were cocultured with 1×10^4 irradiated autologous splenocytes in duplicate for 72 hours. Alpha-fodrin-specific T cell lines (stimulation index >3) were retested using synthetic peptides and were maintained by restimulation at 10–14-day intervals with α -fodrin peptide pulse-irradiated splenocytes. Following a third round of stimulation, LDA revealed cloned T cell lines. Autoreactive T cell lines were maintained by stimulation with IL-2 and feeder cells at 7–10-day intervals.

Flow cytometric analysis. Single cell suspensions were stained with antibodies conjugated to phycoerythrin (PE) (anti-CD3 [Gibco BRL, Grand Island, NY]; anti-CD4, B220) or anti-CD4; fluorescein isothiocyanate (FITC) (anti-CD8, Thy1.2, anti-CD44, anti-CD45RB, anti-Mel-14 [the latter 4 from PharMingen]), and analyzed on an EPICS counter (Coulter, Hialeah, FL). For analysis of intracellular cytokines by flow cytometry, cells ($10^6/\text{ml}$) were activated with immobilized anti-CD3 monoclonal antibody (Cedarlane) for 4 hours. Monensin (Wako) was added at 2 mM, and 2 hours later cells were collected, washed, and permeabilized with 0.1% saponin in phosphate buffered saline at 4°C for 10 minutes. Cells were incubated with FITC-conjugated anti-IL-2 ($8 \mu\text{g/ml}$), PE-conjugated anti-IL-4 ($5 \mu\text{g/ml}$), and FITC-conjugated anti-IFN γ ($1 \mu\text{g/ml}$) and analyzed on an EPICS counter.

Cytotoxicity assay. Cytotoxicity assays were performed as described previously (21), using peptide-pulsed (10 $\mu\text{g/ml}$) mouse salivary gland (MSG) cells labeled with ^{51}Cr sodium chromate as target at a 1:50 target:effector ratio.

Cell transfer. To examine whether autoreactive CD4+ T cells induce autoimmune lesions, cells from T cell line 21-1 (1×10^6) were injected intraperitoneally into irradiated (7.5 Gy) normal NFS/*sld* mice at 4 weeks, and analyses performed at 8 weeks ($n = 7$) and 12 weeks ($n = 8$) after the injection. As controls, 1×10^6 splenic CD4+ T cells nonpulsed or pulsed and with fibronectin fragment peptide (5 $\mu\text{g/ml}$; Sigma, St. Louis, MO) from syngeneic mice were injected intraperitoneally into irradiated (7.5 Gy) NFS/*sld* mice and analyzed in the same manner ($n = 5$ for each).

Measurement of fluid secretion. Measurement of tear and saliva volume in the transferred NFS/*sld* mice was performed by modification of previously described methods (14,21,22).

Western blot analysis. To detect serum autoantibodies against 120-kd α -fodrin antigen (3), samples were solubilized by heating, and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The autoantigen was electrotransferred to nitrocellulose, which was then quenched with 1% powdered milk in borate buffered saline. Nitrocellulose membranes were incubated with testing serum at a 1:200 dilution in borate buffered saline, and then incubated with peroxidase-conjugated horse anti-mouse IgG (Vector) at a 1:1,000 dilution.

RESULTS

Establishment of autoreactive T cell lines. We have previously identified a cleavage product of 120-kd α -fodrin as an important autoantigen in the pathogenesis of primary SS in both humans and rodents (3,4). To determine whether an immune response in mice with experimental SS could be mounted against recombinant α -fodrin protein, the cDNA encoding human α -fodrin (JS-1, 2.7A, and 3'DA) were constructed by inserting cDNA into the *Eco* RI site of pGEX-4T 1, 2, and 3. When we compared, in parallel, the proliferative T cell responses with individual recombinant α -fodrin fusion protein, we detected a significantly increased proliferation in SS mouse LNCs, spleen cells, and tissue-infiltrating T cells stimulated with JS-1 protein (N-terminal portion of α -fodrin) (data not shown). By LDA, we succeeded in isolating 3 strongly proliferative autoreactive T cell lines (clones 21-1, 21-2, and 21-3) from JS-1 peptide (p21)-stimulated LNCs (Figure 1A), but not from control peptide-stimulated cells. The majority of autoreactive T cells were CD4+ cells bearing V β 6 and containing Th1 cytokines such as IL-2 and IFN γ , but not IL-4 (Figure 1B). We confirmed that the autoreactive CD4+ T cell lines had significant cytotoxicity, against MSG cells from NFS/*sld* mice when tested in a ^{51}Cr -release

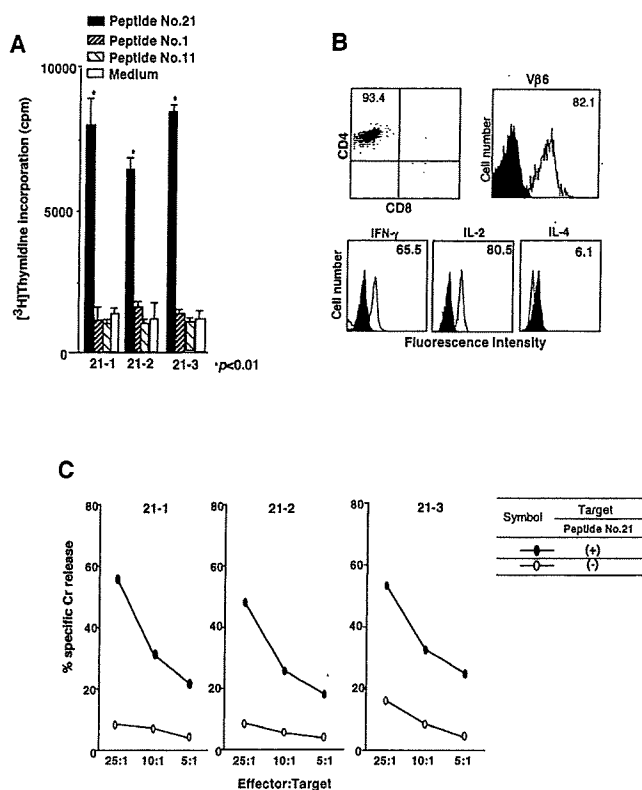


Figure 1. Establishment of autoreactive T cell lines. **A**, Three strongly proliferative T cell lines (21-1, 21-2, 21-3) from JS-1 peptide p21-stimulated lymph node cells were isolated and ^3H -thymidine incorporated measured. Values are the mean and SEM. **B**, Flow cytometric analysis, showing that the majority of these T cells were CD4+ and V β 6+ and contained interleukin-2 (IL-2) and interferon- γ (IFN γ), but not IL-4. **C**, Results of ^{51}Cr -release assay, demonstrating that the autoreactive CD4+ T cell lines had significant cytotoxicity when tested against mouse salivary gland cells. Values are the mean from triplicate studies.

assay (Figure 1C). In contrast, the autoreactive T cells did not kill major histocompatibility complex (MHC)-matched targets of newborn keratinocytes (data not shown).

The TCR V β usage and the CDR3 sequences of 3 autoreactive T cell lines were determined by RT-PCR amplification and sequencing of the PCR products. Notably, in some cases these sequences (SISAETL and SMQN) were homologous to V-D-J β sequences of the T cells from affected glands of mice with experimental SS at 8 weeks (Figure 2).

Development of autoimmune lesions after adoptive transfer of autoreactive CD4+ T cells. To analyze whether the autoreactive T cells cause autoimmune lesions, cells from CD4+ T cell line 21-1 (1×10^6) were transferred intraperitoneally into irradiated (7.5 Gy) normal NFS/*sld* mice at 4 weeks. Organ-specific auto-

	Vβ6	N-D-N	Jβ		
T cells infiltrated in salivary gland					
	TGTGCCAGC	AGTATAGCGAGAA	TTGGTCCCGGGCACCAGGCTGGTTF	2.1	
	C A S	<u>S H Q N</u>	F G P G T R L G F		
	TGTGCCAGC	AGTATAAGTGCAGAAACGCTG	TATTTGGTCCCGGGCACCAGGCTGGTTF	2.3	
	C A S	<u>S X S A E R L</u>	Y F G A G T R L S V		
Autoreactive T cell lines					
21-1	TGTGCCAGC	AGTATAAGTGCAGAAACGCTG	TATTTGGTCCCGGGCACCAGGCTGGTTF	2.3	8/10
	C A S	<u>S X S A E R L</u>	Y F G A G T R L S V		
	TGTGCCAGC	AGTATAGCAGTA	TTGGTCCCGGGCACCAGGCTGGTTF	2.1	1/10
	C A S	<u>S H T V</u>	F G P G T R L G F		
	TGTGCCAGC	AGTATAGGZMAGATATGACAG	TACTTCGGTCCCGGGCACCAGGCTGGTTF	2.6	1/10
	C A S	<u>S I G H R Y E Q</u>	Y F G P G T R L T V		
	TGTGCCAGC	AGCCCGGAGAAACGCTG	TATTTGGTCCCGGGCACCAGGCTGGTTF	2.6	1/10
	C A S	<u>S F A E T F</u>	Y F G P X T R L T V		
21-2	TGTGCCAGC	AGTATAAGTGCAGAAACGCTG	TATTTGGTCCCGGGCACCAGGCTGGTTF	2.3	7/10
	C A S	<u>S X S A E R L</u>	Y F G A G T R L S V		
	TGTGCCAGC	AGTATAGGTTGAAACG	TACTTCGGTCCCGGGCACCAGGCTGGTTF	2.5	1/10
	C A S	<u>S I H V E Q</u>	Y F G P G T R L T V		
	TGTGCCAGT	AGTACCGGNCCTGGGGTCAAGACACCCAG	TACTTCGGTCCCGGGCACCAGGCTGGTTF	2.5	1/10
	C A S	<u>S T G L G V Q D T Q</u>	Y F G P G T R L L V		
	TGTGCCAGC	AGCCCGGAGAAACGCTG	ATTTTGGTCCCGGGCACCAGGCTGGTTF	2.6	1/10
	C A S	<u>S F A E T F</u>	Y F G L X T R L T V		
21-3	TGTGCCAGC	AGTATAGCAGTA	TTGGTCCCGGGCACCAGGCTGGTTF	2.1	7/10
	C A S	<u>S H Q N</u>	F G P G T R L G F		
	TGTGCCAGC	AGTATAAGTGCAGAAACGCTG	TATTTGGTCCCGGGCACCAGGCTGGTTF	2.3	1/10
	C A S	<u>S X S A E R L</u>	Y F G A G T R L S V		
	TGTGCCAGT	AGTACCGGNCCTGGGGTCAAGACACCCAG	TACTTCGGTCCCGGGCACCAGGCTGGTTF	2.5	1/10
	C A S	<u>S T G L G V Q D T Q</u>	Y F G P G T R L L V		
	TGTGCCAGC	AGTACCGGNCCTGGGGTCAAGACACCCAG	TACTTCGGTCCCGGGCACCAGGCTGGTTF	2.6	1/10
	C A S	<u>S R D G Y E Q</u>	Y F G P G T R L T V		

Figure 2. T cell receptor Vβ gene usage and third complementarity-determining region (CDR3) sequences of infiltrating T cells in NFS/*sld* mice that had undergone thymectomy on day 3 after birth and of α-fodrin p21-specific lines (21-2, 21-2, 21-3), determined by reverse transcriptase-polymerase chain reaction amplification and sequencing of the polymerase chain reaction products. The Vβ6-CDR3 sequences were homologous between the T cells infiltrating salivary glands and autoreactive T cell lines (underlined).

immune lesions developed exclusively in the salivary and lacrimal glands at 8 weeks (n = 7) and 12 weeks (n = 8) after the injection with autoreactive T cell line 21-1, while a transfer of splenic CD4+ T cells pulsed with fibronectin fragment peptide did not induce any lesions (Figure 3A). Histopathologic examination revealed no

inflammatory lesions in other organs including the liver, pancreas, adrenal glands, and reproductive organs of mice treated with the autoreactive T cell line. This suggests that α-fodrin-reactive CD4+ T cells are pathogenic in vivo.

The majority of tissue-infiltrating cells in the salivary and lacrimal glands of mice that underwent adoptive transfer of autoreactive CD4+ T cells were positive for CD4 and Vβ6, but not for CD8 or Vβ8 (Figure 3B). Very few B220+ B cells were present in inflammatory lesions (results not shown). A large proportion of Th1 cytokine-positive cells (IL-2, IFNγ), but not IL-4-positive cells, was detected in the salivary glands from autoreactive T cell line-treated mice (Figure 4A). Isotype-matched controls were all negative. Moreover, the autoimmune lesions were accompanied by significantly decreased secretion of saliva and tears (Figure 4B). Serum autoantibody production against JS-1 protein could not be detected in adoptively transferred mice (Figure 4C). T cell line-treated mice showed a significant increase of autoantigen (JS-1 and p21)-specific T cell proliferation in the spleen cells, while no responses against ovalbumin or lysozyme were observed (Figure 5A). The activation markers CD44^{high}, CD45RB^{low}, and Mel-14^{low} were significantly up-regulated in LNCs gated on CD4 from adoptively transferred mice (Figure 5B). Moreover, we found that CD4+ T cells isolated from LNCs of the treated mice had significant cytotoxicity when tested against MSG

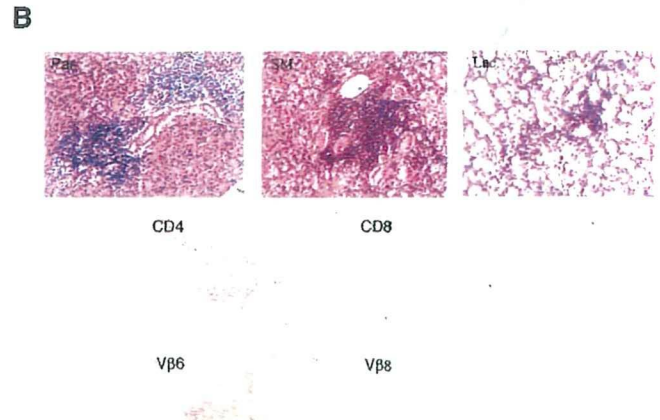
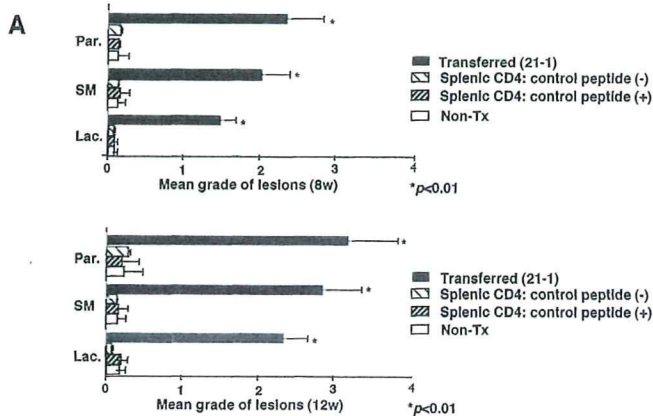


Figure 3. Adoptive transfer of autoreactive T cell line 21-1 into normal syngeneic recipients. **A**, Autoimmune lesions in the salivary and lacrimal (Lac.) glands developed at 8 weeks (n = 7) and 12 weeks (n = 8) after intraperitoneal injection with cells from T cell line 21-1 (1 × 10⁶) into irradiated (7.5 Gy) normal NFS/*sld* mice, but not in controls. Values are the mean and SEM. Par. = parotid gland; SM = submandibular gland; Non-Tx = nonthymectomized. **B**, Representative histologic features in adoptively transferred mice at 12 weeks. The majority of infiltrating lymphocytes were positive for CD4 and Vβ6, but not for CD8 or Vβ8.

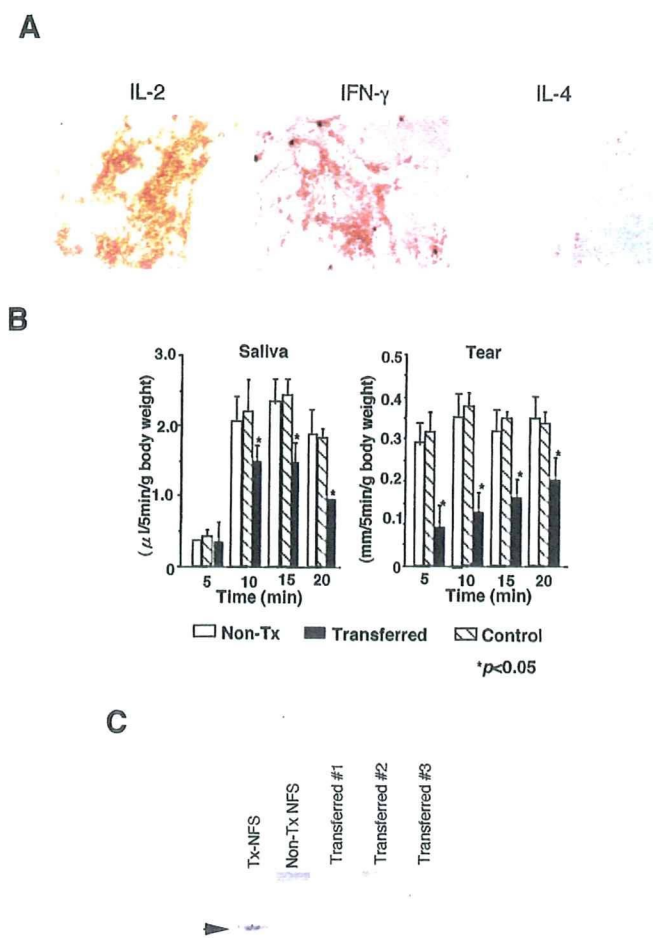


Figure 4. A, Immunohistologic features in adoptively transferred mice. A large proportion of infiltrating cells in the salivary glands were positive for Th1 cytokines such as interleukin-2 (IL-2) and interferon- γ (IFN γ), but not IL-4. B, Mean and SEM saliva and tear volume (n = 5 per group) in adoptively transferred mice, control-treated mice, and nonthymectomized (Non-Tx) mice. C, Western blot analysis. Production of autoantibodies to 120-kD α -fodrin was not detected in sera from 3 different adoptively transferred mice.

cells from NFS/sld mice in a ⁵¹Cr-release assay (Figure 5C).

DISCUSSION

Autoreactive T cells are conventionally regarded to be eliminated by negative selection in the thymus or by the induction of peripheral tolerance (23,24). The results described here demonstrate that α -fodrin-reactive CD4+ T cells can induce autoimmune exocri-

nopathy in normal syngeneic mice. Although the specificity of cytotoxic T lymphocyte (CTL) function has been an important issue in organ-specific autoimmune response, the mechanisms responsible for tissue destruction have not been elucidated. In type 1 diabetes mellitus, the role of environmental factors (25,26), the nature of the initiating inflammatory cell (27,28), and the identity of the inciting antigen(s) (29,30) have all been vigorously debated. We have previously identified a cleavage product of 120-kd α -fodrin as an important autoantigen in the pathogenesis of primary SS in both humans and rodents (3,4). We detected significantly increased proliferation of lymph node cells, spleen cells, and tissue-infiltrating T cells from model mice (3d-Tx NFS/sld) stimulated with JS-1 protein (N-terminal portion of α -fodrin).

We succeeded in isolating 3 strongly proliferative autoreactive T cell lines (21-1, 21-2, and 21-3) from JS-1 peptide (p21)-stimulated LNCs. The majority of autoreactive T cells were CD4+ T cells bearing V β 6 and containing Th1 cytokines such as IL-2 and IFN γ , but not IL-4. Of importance is that the autoreactive CD4+ T cell lines had significant cytotoxicity when tested against MSG cells in a ⁵¹Cr-release assay. Furthermore, the TCR V β usage and the CDR3 sequences of 3 autoreactive T cell lines were homologous to VDJ β sequences of the T cells from affected glands of mice with experimental SS. (Figure 2).

Previous studies have suggested that clonally expanded T cell populations with restricted usage of TCR gene segments may be essential for the development of autoimmune diseases including SS (31,32). However, the basis for TCR repertoire selection initiating autoimmunity has not yet been fully understood. It should be noted that in this study, infiltrating T cell sequences that are similar to, and in some cases match, the sequences of the autoreactive T cell lines were found (underlined in Figure 2). Previous work has demonstrated that dual TCR T cells may rescue autoreactive T cells from negative selection in the thymus (33). Our data imply that the established autoreactive T cells are found in the common TCR repertoire (V β 6-CDR3: SISAETL). This notion is supported by work by Basu et al (34) which demonstrates the binding of 2 separate ligands, a self peptide (arthritic peptide), and a foreign epitope, on distinct MHC areas by T cells bearing a single TCR.

In the analysis of whether the autoreactive T cells cause autoimmune lesions, we found that organ-specific autoimmune lesions developed exclusively in the salivary and lacrimal glands at 8 and 12 weeks after the intra-

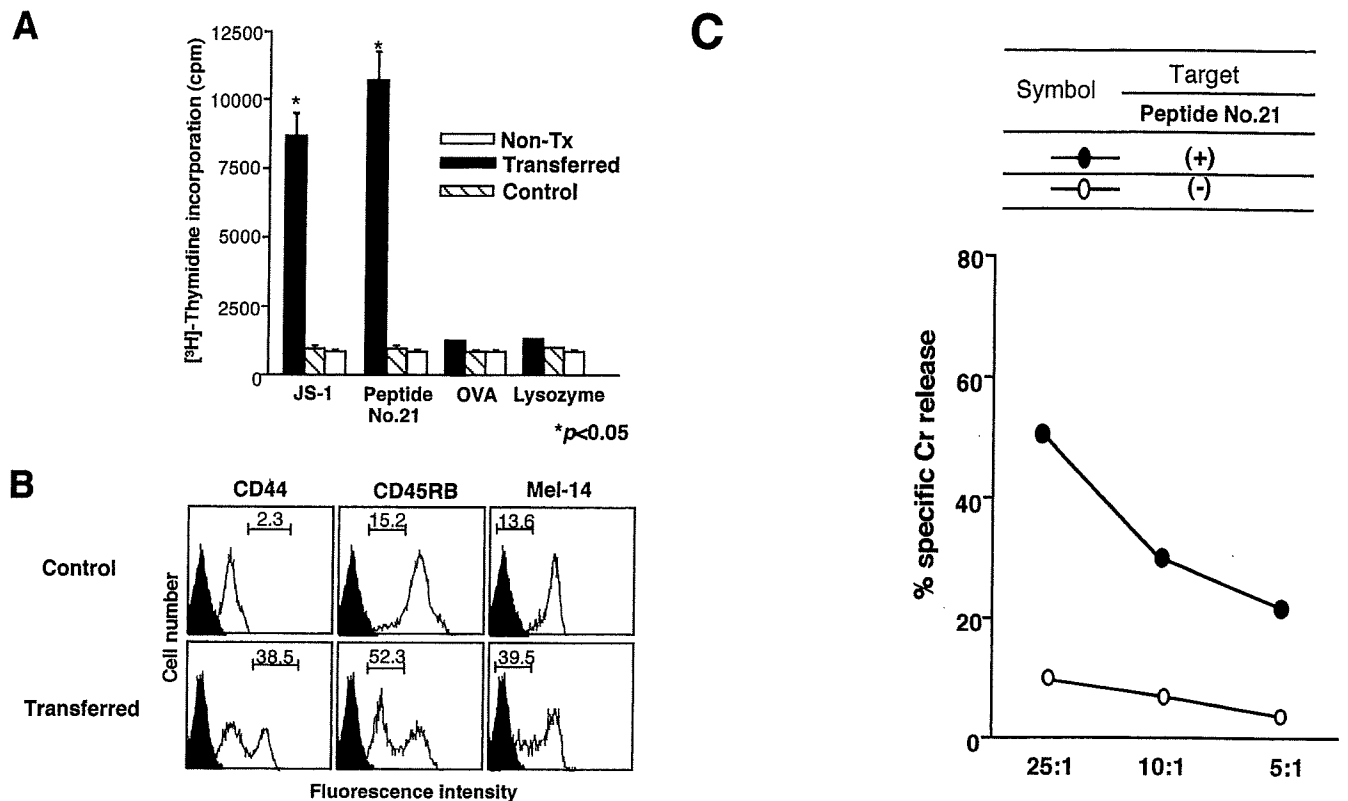


Figure 5. A, T cell proliferation in spleen cells. Adoptively transferred mice showed a significant increase of autoantigen (JS-1 and peptide 21)-specific T cell proliferation, while no responses against ovalbumin (OVA) or lysozyme were observed. Values are the mean and SEM of triplicate experiments. Non-Tx = nonthymectomized. B, Flow cytometric analysis, showing that the activation markers CD44^{high}, CD45RB^{low}, and Mel-14^{low} were significantly up-regulated in lymph node cells gated on CD4 from adoptively transferred mice. C, Results of ⁵¹Cr-release assay, demonstrating that CD4⁺ T cells isolated from lymph node cells of adoptively transferred mice had significant cytotoxicity against salivary gland cells from NFS/sld mice. Values are the mean from triplicate studies.

peritoneal injection with autoreactive CD4⁺ T cells. Adoptively transferred mice showed a significant increase of autoantigen-specific T cell proliferation in the spleen cells, while no responses against ovalbumin or lysozyme were observed. The activation markers were significantly up-regulated in LNCs gated on CD4 from adoptively transferred mice, and CD4⁺ T cells isolated from LNCs of transferred mice had significant cytotoxicity against MSG cells when tested in a ⁵¹Cr-release assay. These data indicate that the autoreactive CD4⁺ T cells recognizing α -fodrin autopeptide are essentially pathogenic for the development of organ-specific autoimmune lesions in murine SS. Since serum production of autoantibodies against α -fodrin autoantigen could not be detected in transferred mice, it is possible that the adoptively transferred disease in these experiments may be entirely dependent on T cell-mediated immune responses. Thus, a critical autoreactive CD4⁺ T cell function should be operative in the initial stages of the

disease, because the T cells in established lesions show strong proliferative activity and secrete Th1 cytokines. Previous investigations have demonstrated the accumulation of antigen-reactive T cells at the site of the inflammation in several human autoimmune diseases as well as in murine models of human autoimmune diseases (35,36).

In conclusion, we have demonstrated that α -fodrin-specific autoreactive CD4⁺ T cell lines can be established from α -fodrin peptide p21-stimulated LNCs, and that the autoreactive T cells have significant cytotoxicity against MSG cells when tested in a CTL assay. Moreover, we confirmed the development of autoimmune lesions, quite similar to those found in SS, into normal syngeneic recipients, using autoreactive CD4⁺ T cells. The results of this study provide evidence of an essential role for autoreactive CD4⁺ T cells specific for a self peptide in the development of organ-specific autoimmune disease in SS.