gen prevents bone loss through multiple effects on bone marrow and bone cells, which result in decreased OC formation (50), increased OC apoptosis (51), and decreased capacity of mature OCs to resorb bone (52). Because it was also demonstrated by direct evidence that treatment with estrogens suppressed RANKL-mediated OC formation (53), it is possible that their contribution to the increased osteoclastogenesis and the bone loss has been induced by estrogen deficiency.

In conclusion, we have demonstrated that activation of $CD4^+$ T cells bearing RANKL induced by an estrogen deficiency may play an important role on acceleration of autoimmune arthritis, and estrogenic action appears to influence joint destruction associated with RANKL-mediated osteoclastogenesis in a murine model for RA.

## Acknowledgments

Received November 12, 2003. Accepted January 8, 2004.

Address all correspondence and requests for reprints to: Dr. Yoshio Hayashi, Department of Pathology, Tokushima University School of Dentistry, 3 Kuramotocho, Tokushima 770-8504, Japan. E-mail: hayashi@dent.tokushima-u.ac.jp.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

## References

1. **Lahita RG, Bradlow L, Fishman J, HG Kunkel HG** 1982 Estrogen metabolism in systemic lupus erythematosus: patients and family members. Arthritis Rheum 25:843–846
2. **Zurier RB** 1987 Systemic lupus erythematosus. In: Lahita RG, ed. New York: Wiley Publishers; 541–554
3. **Daniels T, Whitcher JP** 1994 Association of patients of labial salivary gland inflammation with keratoconjunctivitis sicca. Analysis of 618 patients with suspected Sjögren's syndrome. Arthritis Rheum 37:869–877
4. **Davidson A, Diamond B** 2001 Autoimmune diseases. N Engl J Med 345:340–350
5. **Yamamura Y, Gupta R, Morita Y, He X, Pai R, Endres J, Freiberg A, Chung K, Fox DA** 2001 Effector function of resting T cells: activation of synovial fibroblasts. J Immunol 166:2270–2275
6. **Park CC, Morel JC, Amin MA, Connors MA, Harlow LA, Koch AE** 2001 Evidence of IL-18 as a novel angiogenic mediator. J Immunol 167:1644–1653
7. **van den Berg WB** 2001 Arguments for interleukin 1 as a target in chronic arthritis. Ann Rheum Dis 59:81–84
8. **Pap T, Shigeyama Y, Kuchen S, Fernihough JK, Simmen B, Gay RE, Billingham M, Gay S** 2000 Differential expression pattern of membrane-type matrix metalloproteinases in rheumatoid arthritis. Arthritis Rheum 43:1226–1232
9. **Takayanagi H, Iizuka H, Juji T, Nakagawa T, Yamamoto A, Miyazaki T, Koshihara Y, Oda H, Nakamura K, Tanaka S** 2000 Involvement of receptor activator of nuclear factor κB ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis. Arthritis Rheum 43:259–269
10. **Kotake S, Udagawa N, Hakoda M, Mogi M, Yano K, Tsuda E, Takahashi K, Furuya T, Ishiyama S, Kim KJ, Saito S, Nishikawa T, Takahashi N, Togari A, Tomatsu T, Suda T, Kamatani N** 2001 Activated human T cells directly induce osteoclastogenesis from human monocytes: possible role of T cells in bone destruction in rheumatoid arthritis patients. Arthritis Rheum 44:1003–1012
11. **Sabokbar A, Fujikawa Y, Neale S, Murray DW, Athanasou NA** 1997 Human arthroplasty derived macrophages differentiate into osteoclastic bone resorbing cells. Ann Rheum Dis 56:414–420
12. **Rothe L, Collin-Osdoby P, Chen Y, Sunyer T, Chaudhary L, Tsay A, Goldring S, Avioli L, Osdoby P** 1998 Human osteoclasts and osteoclast-like cells synthesize and release high basal and inflammatory stimulated levels of the potent chemokine interleukin-8. Endocrinology 139:4353–4363
13. **Gravallese EM, Manning C, Tsay A, Naito A, Pan C, Amento E, Goldring SR** 2000 Synovial tissue in rheumatoid arthritis is a source of osteoclast differentiation factor. Arthritis Rheum 43:250–258
14. **Wong B, Rho J, Arron J, Robinson E, Orlinick J, Chao M, Kalachikov S, Cayani E, Bartlett 3rd FS, Frankel WN, Lee SY, Choi Y** 1997 TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. J Biol Chem 272:25190–25194
15. **Wong BR, Josien R, Choi Y** 1999 TRANCE is a TNF family member that regulates dendritic cell and osteoclast function. J Leukoc Biol 65:715–724
16. **Josien R, Wong BR, Li HL, Steinman RM, Choi Y** 1999 TRANCE, a TNF family member, is differentially expressed on T cell subsets and induces cytokine production in dendritic cells. J Immunol 162:2562–2568
17. **Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, Teepe MC, DuBose RF, Cosman D, Galibert L** 1997 A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. Nature 390:175–179
18. **Josien R, Li HL, Ingulli E, Sarma S, Wong BR, Vologodskaia BM, Steinman RM, Choi Y** 2000 TRANCE, a tumor necrosis factor family member, enhances the longevity and adjuvant properties of dendritic cells in vivo. J Exp Med 191:495–502
19. **Bachmann MF, Wong BR, Josien R, Steinman RM, Oxenius A, Choi Y** 1999 TRANCE, a tumor necrosis factor family member critical for CD40 ligand-independent T helper cell activation. J Exp Med 189:1025–1031
20. **Wong BR, Josien R, Lee SY, Sauter B, Li HL, Steinman RM, Choi Y** 1997 TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. J Exp Med 186:2075–2080
21. **Cohen PL, Eisenberg RA** 1991 Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. Annu Rev Immunol 9:243–269
22. **Theofilopuolos AN, Dixon FJ** 1985 Murine models of systemic lupus erythematosus. Adv Immunol 37:269–390
23. **Merino R, Iwamoto M, Fossati L, Izui S** 1993 Polyclonal B cell activation arises from different mechanisms in lupus-prone (NZBx NZW)F1 and MRL/MpJ-lpr/lpr mice. J Immunol 151:6509–6516
24. **Edwards 3rd CK, Zhou T, Zhang J, Baker TJ, De M, Long RE, Borcherding DR, Bowlin TL, Bluethmann H, Mountz JD** 1996 Inhibition of superantigen-induced proinflammatory cytokine production and inflammatory arthritis in MRL-lpr/lpr mice by a transcriptional inhibitor of TNF-α. J Immunol 157:1758–1772
25. **Feeney AJ, Lawson BR, Kono BDH, Theofilopoulos AN** 2001 Terminal deoxynucleotidyl transferase deficiency decreases autoimmune disease in MRL-$Fas^{lpr}$ mice. J Immunol 167:3486–3493
26. **Fields ML, Sokol CL, Eaton-Bassiri A, Seo S, Madaio MP, Erikson J** 2001 Fas/fas ligand deficiency results in altered localization of anti-double-stranded DNA B cells and dendritic cells. J Immunol 167:2370–2378
27. **Kageyama Y, Koide Y, Yoshida A, Uchijima M, Arai T, Miyamoto S, Ozeki T, Hiyoshi M, Kushida K, Inoue T** 1998 Reduced susceptibility to collagen-induced arthritis in mice deficient in IFN-γ receptor. J Immunol 161:1542–1548
28. **Saegusa K, Ishimaru N, Yanagi K, Haneji N, Nishino M, Azuma M, Saito I, Hayashi Y** 2000 Autoantigen-specific $CD4^+CD28^{low}$ T cell subset prevents autoimmune exocrinopathy in murine Sjögren's syndrome. J Immunol 165:2251–2257
29. **Takahashi N, Yamana H, Yoshiki S, Roodman GD, Mundy GR, Jones SJ, Boyde A, Suda T** 1998 Osteoclast-like cell formation and its regulation by osteotropic hormones in mouse bone marrow cultures. Endocrinology 122:1373–1382
30. **Udagawa N, Takahashi N, Akatsu T, Tanaka H, Sasaki T, Nishihara T, Koga T, Martin TJ, Suda T** 1990 Origin of osteoclast: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. Proc Natl Acad Sci USA 87:7260–7264
31. **Choy EH, Panayi GS** 2001 Cytokine pathways and joint inflammation in rheumatoid arthritis. N Engl J Med 344:907–916
32. **Lahita RG** 1985 Sex steroids and the rheumatic diseases. Arthritis Rheum 28:121–126
33. **Bateman A, Singh A, Kral T** 1989 The immune-hypothalamic-pituitary-adrenal axis. Endocr Rev 10:92–112
34. **Grossman C** 1989 Possible underlying mechanisms of sexual dimorphism in the immune response, fact and hypothesis. J Steroid Biochem 34:241–251
35. **Holmdahl R, Jansson L, Meyerson B, Klareskog L** 1987 Oestrogen induced suppression of collagen arthritis: I. Long term oestradiol treatment of DBA/1 mice reduces severity and incidence of arthritis and decreases the anti-type II collagen immune response. Clin Exp Immunol 70:372–378
36. **Masuzawa T, Miura C, Onoe Y, Kusano K, Ohta H, Nozawa S, Suda T** 1994 Estrogen deficiency stimulates B lymphopoiesis in mouse bone marrow. J Clin Invest 94:1090–1097
37. **Verthelyi D, Ahmed SA** 1994 17β-estradiol, but not 5α-dihydrotestosterone, augments antibodies to double-stranded deoxyribonucleic acid in nonautoimmune C57BL/6 mice. Endocrinology 135:2615–2622
38. **Brick J, Walker S, Wise K** 1988 Hormone control to calf thymus nuclear extract (CTE) and DNA in MRL/lpr and MRL/+/+ mice. Clin Immunol Immunopathol 46:68–81
39. **Ralston SH** 1994 Analysis of gene expression in human bone biopsies by polymerase chain reaction: evidence for enhanced cytokine expression in postmenopausal osteoporosis. J Bone Miner Res 9:883–890
40. **Jilka RL, Hangoc G, Girasole G, Passeri G, Manolagas DC** 1992 Increased osteoclast development after estrogen loss: mediation by interleukin-6. Science 257:88–91

41. **Fox HS, Bond BL, Parslow TG** 1991 Estrogen regulates the IFN-γ promoter. J Immunol 146:4362–4367
42. **Cenci S, Toraldo G, Weitzmann N, Roggia C, Gao Y, Qian WP, Sierra O, Pacifici R** 2003 Estrogen deficiency induces bone loss by increasing T cell proliferation and lifespan through IFN-γ-induced class II transactivator. Proc Natl Acad Sci USA 100:10405–10410
43. **Weitzmann MN, Cenci S, Rifas L, Haug J, Dipersio J, Pacifici R** 2001 T cell activation induces human osteoclast formation via receptor activator of nuclear factor kappa B ligand-dependent and -independent mechanisms. J Bone Miner Res 16:328–337
44. **Romas E, Bakharevski O, Hards DK, Kartsogiannis V, Quinn JM, Ryan PF, Martin TJ, Gillespie MT** 2000 Expression of osteoclast differentiation factor at sites of bone erosion in collagen-induced arthritis. Arthritis Rheum 43:821–826
45. **Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, Capparelli C, Li J, Elliott R, McCabe S, Wong T, Campagnuolo G, Moran E, Bogoch ER, Van G, Nguyen LT, Ohashi PS, Lacey DL, Fish E, Boyle WJ, Penninger JM** 1999 Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature 402:304–309
46. **Eghbali-Fatourechi G, Khosla S, Sanyal A, Boyle WJ, Lacey DL, Riggs BL** 2003 Role of RANK ligand in mediating increased bone resorption in early postmenopausal women. J Clin Invest 111:1221–1230
47. **Kong YY, Penninger JM** 2000 Molecular control of bone remodeling and osteoporosis. Exp Gerontol 35:947–956
48. **Rodan GA, Martin TJ** 2000 Therapeutic approaches to bone diseases. Science 289:1508–1514
49. **Merkel KD, Erdmann JM, McHugh KP, Abu-Amer Y, Ross FP, Teitelbaum SL** 1999 Tumor necrosis factor-α mediates orthopedic implant osteolysis. Am J Pathol 154:203–210
50. **Pacifici R** 1996 Estrogen, cytokines and pathogenesis of postmenopausal osteoporosis. J Bone Miner Res 11:1043–1051
51. **Hughes DE, Dai A, Tiffee JC, Li HH, Mundy GR, Boyce BF** 1996 Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-β. Nat Med 2:1132–1136
52. **Oursler MJ, Osdoby P, Pyfferoen J, Riggs BL, Spelsberg TC** 1991 Avian osteoclasts as estrogen target cells. Proc Natl Acad Sci USA 88:6613–6617
53. **Shevde NK, Bendixen AC, Dienger KM, Pike JW** 2000 Estrogens suppress RANK ligand-induced osteoclast differentiation via a stromal cell independent mechanism involving b-Jun repression. Proc Natl Acad Sci USA 97:7829–7834

*Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

# Apoptosis and estrogen deficiency in primary Sjögren syndrome

Yoshio Hayashi, Rieko Arakaki and Naozumi Ishimaru

**Purpose of review**

Primary Sjögren syndrome is an autoimmune disorder characterized by lymphocytic infiltrates and destruction of the salivary and lacrimal glands, and systemic production of autoantibodies to the ribonucleoprotein particles SS-A/Ro and SS-B/La. The purpose of this review is to discuss recent advances in the pathogenesis of primary Sjögren syndrome.

**Recent findings**

Although several candidate autoantigens including α-fodrin have been reported in Sjögren syndrome, the pathogenic roles of the autoantigens in initiation and progression of SS are still unclear. It is possible that individual T cells activated by an appropriate self antigen can proliferate and form a restricted clone. Recent evidence suggests that the apoptotic pathway plays a central role in tolerizing T cells to tissue-specific self antigen, and may drive the autoimmune phenomenon. Cleavage of certain autoantigens during apoptosis may reveal immunocryptic epitopes that could potentially induce autoimmune response. The studies reviewed imply that Fas-mediated cytotoxicity and caspase-mediated α-fodrin proteolysis are involved in the progression of tissue destruction in Sjögren syndrome. Fas ligand (FasL), and its receptor Fas are essential in the homeostasis of the peripheral immune system. It can be considered that a defect in activation-induced cell death of effector T cells may result in the development of autoimmune exocrinopathy in Sjögren syndrome.

**Summary**

Although the mechanisms by which estrogen deficiency influences autoimmune lesions remain unclear, it is possible that antiestrogenic actions might be a potent factor in the formation of pathogenic autoantigens.

**Keywords**

Sjögren syndrome, apoptosis, activation-induced cell death, estrogen deficiency

Curr Opin Rheumatol 16:522–526. © 2004 Lippincott Williams & Wilkins.

Department of Pathology, Tokushima University School of Dentistry, Tokushima, Japan

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

Correspondence to Yoshio Hayashi, Department of Pathology, Tokushima University School of Dentistry, 3 Kuramotocho, Tokushima 770, Japan
Tel: 81 88 633 7327; fax: 81 88 633 7327;
e-mail: hayashi@dent.tokushima-u.ac.jp



Current Opinion in Rheumatology 2004, 16:522–526

Abbreviations

| | |
|---|---|
| AICD | activation-induced cell death |
| FasL | Fas ligand |
| SS | Sjögren syndrome |
| TCR | T-cell antigen receptor |


1040–8711

## Introduction

Organ-specific autoimmune diseases are characterized by tissue destruction and functional decline due to autoreactive T cells that escape self-tolerance [1,2]. Sjögren syndrome (SS) is a T-cell-mediated autoimmune disorder characterized by lymphocytic infiltrates and destruction of the salivary and lacrimal glands, and systemic production of autoantibodies to the ribonucleoprotein particles SS-A/Ro and SS-B/La [3,4]. Autoreactive T cells bearing CD4 molecule may recognize unknown autoantigen triggering autoimmunity in the salivary and lacrimal glands, leading to clinical symptoms of dryness of the mouth and eyes (sicca syndrome) [5]. It is now evident that the interaction of Fas with FasL regulates a large number of pathophysiological processes of apoptosis including autoimmune diseases [6]. Recent studies have now confirmed the observation that apoptotic cells in various cell types are implicated as the source of autoantigen when stimulated with different proapoptotic stimuli [7,8]. Although cleavage of certain autoantigens during apoptosis may reveal immunocryptic epitopes that could potentially induce autoimmune responses in systemic autoimmune diseases [9], accumulated evidences suggest an important role of apoptosis in disease pathogenesis of Sjögren syndrome [10].

## Apoptotic cells in Sjögren syndrome

Recent studies have suggested that the Fas-Fas ligand (FasL) system plays a major role on the induction of apoptosis in target organs with autoimmune diseases such as autoimmune gastritis, Hashimoto thyroiditis, and rheumatoid arthritis [11,12]. 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 thyroiditis [13]. In contrast, expression of Fas by pancreatic β cells has been shown to have a major influence on the susceptibility of tissue destruc-

tion in nonobese diabetic (NOD) mice to diabetes [14,15•]. Since it was reported that Fas expression was observed in the salivary gland cells with human Sjögren syndrome [16], it was likely that Fas-mediated apoptosis may contribute to tissue destruction in the salivary glands with Sjögren syndrome. A cleavage product of 120-kDa α-fodrin was identified as an important autoantigen in human Sjögren syndrome besides NFS/sld murine model for Sjögren syndrome [17]. α-Fodrin is a ubiquitous, calmodulin-binding protein [18] found to be cleaved by calcium-activated protease (calpain) in apoptotic T cells, and by calpain or caspase 3 [19] in anti-Fas-stimulated Jurkat cells or neuronal apoptosis [20]. It was demonstrated that the fodrin α subunit is cleaved in association with apoptosis, and the 120-kDa fragment is a breakdown product of the mature form of 240-kDa fodrin-α subunit [20,21]. Previous studies have demonstrated evidence that caspase 3 is required for α-fodrin cleavage during apoptosis [21]. In Jurkat cells, caspase 3-like proteases have been reported to cleave α-fodrin and poly (ADP-ribose) polymerase [21]. The observation that ubiquitously expressed autoantigens (e.g., α-fodrin, La, and nuclear mitotic apparatus protein) in Sjögren syndrome are specifically cleaved by granzyme B strongly suggests that a common biochemical event (novel autoantigen cleavage during granule-induced epithelial cell death) is responsible for selecting the unconnected group of molecules [22].

In vitro studies demonstrated that apoptotic mouse salivary gland (MSG) cells result in a specific α-fodrin cleavage into 120 kDa, and preincubation with caspase-inhibitor peptides blocked α-fodrin cleavage [23]. A significant increase of TUNEL$^+$-apoptotic epithelial duct cells in the salivary glands was detected in NFS/sld Sjögren syndrome mouse model. MSG cells constitutively express Fas with high proportion, and tissue-infiltrating CD4$^+$ T cells isolated from the salivary gland tissues bear a large proportion of FasL. Importantly, the tissue-infiltrating CD4$^+$ T cells, but not CD8$^+$ T cells, are responsible for tissue destruction as judged by *in vitro* $^{51}$Cr release cytotoxic assay against MSG cells *in vitro*. Although it has been reported that Fas-induced apoptosis seems to be the major killing pathway of the CD4$^+$ cytotoxic T cells [24], one mechanism by which activated CD4$^+$ T cells induce cytotoxicity towards salivary gland cells in Sjögren syndrome is Fas based. *In vivo* treatment with caspase-inhibitors, z-VAD-fmk and DEVD-CHO, into murine model results in dramatically inhibitory effects on the development of autoimmune lesions, and in restoration of sicca syndrome [23]. There is increasing evidence that the cascade of caspases is a critical component of the cell death pathway [25,26], and a few proteins have been found to be cleaved during apoptosis. These include poly (ADP-ribose) polymerase, a small U1 nuclear ribonucleoprotein, and α-fodrin, which were subsequently identified as substrates for caspases [27,28]. The development of autoimmune exocrinopathy in Sjögren syndrome appears to be dependent on autoantigen cleavage through caspase cascade, and caspase-inhibitors might provide a new therapeutic option directed at reducing tissue damage.

## T cell apoptosis in Sjögren syndrome

Activation-induced cell death (AICD) is a well-known mechanism of peripheral T-cell tolerance that depends upon an interaction between Fas and FasL [29]. AICD plays a central role, especially in killing autoreactive T cells and in preventing autoimmune responses [30]. It has been reported that activation of T-cell clones induces FasL expression, and AICD in autoreactive T cells *in vivo* has been proposed to limit the expansion of an immune response by eliminating effector cells [31••]. Although it can be considered that a defect in AICD of effector T cells may result in the development of autoimmune disease [32], an *in vivo* role of organ-specific autoantigen for AICD is entirely unclear. Because the administration of a soluble form of anti-FasL antibody (FLIM58) results in severe destructive autoimmune exocrinopathy in a murine model of Sjögren syndrome [33], it is possible that an organ-specific autoantigen may play an important role on down-regulation of AICD. A high titer of serum autoantibodies against 120-kD α-fodrin autoantigen was detected in the FLIM58-treated mice, and splenic T-cell culture supernatants contained high level of interferon-γ. FasL-mediated AICD is down-regulated by autoantigen stimulation in spleen cells from the murine Sjögren syndrome model, but not from Fas-deficient MRL/lpr mice and FasL-deficient MRL/gld mice. FasL undergo matrix metalloproteinase–mediated proteolytic processing in their extracellular domains, resulting in the release of soluble trimeric ligands (soluble FasL [sFasL]). In this case, the processing of sFasL occurs in autoantigen-specific CD4$^+$ T cells, and a significant increase in expressions of metalloproteinase-9 mRNA was observed in spleen cells from Sjögren syndrome mouse model [33]. The increased generation of sFasL inhibits the normal AICD process, leading to the proliferation of effector CD4$^+$ T cells in the murine SS model. Previous studies have demonstrated that CD4$^+$ T cells are susceptible to AICD induced through T-cell receptor–mediated recognition of allogeneic MHC class II molecules, supporting the notion that AICD can be triggered in activated T cells through the T-cell receptor–mediated recognition of antigen [34,35]. Mice or human individuals lacking functional Fas or FasL display profound lymphoproliferative reactions associated with autoimmune disorders [36]. In proteoglycan-induced arthritis, CD4$^+$ T cells proliferate at a high rate in response to proteoglycan stimulation, and exhibit a Th1-type response [37]. These observations have suggested that a defect in AICD of autoreactive Th1 cells may contribute to the pathogenesis of Sjögren syndrome.

## Estrogen deficiency in Sjögren syndrome

Sex hormones influence both humoral and cell-mediated immune response, and estrogen is one of the potential factors in this immunologic dimorphism [38,39]. Estrogenic action has been suggested to be responsible for the strong female preponderance of autoimmune diseases including systemic lupus erythematosus and SS [40,41]. Although a number of autoimmune diseases are known to develop in postmenopausal women, the mechanisms by which estrogen deficiency influences autoimmune lesions remain unclear. Previous reports indicate that the increase in autoantibody production as a result of estrogen deficiency is mediated by cytokines such as interleukin-6, interferon-γ (interferon-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 and activation of human monocytes [42–44]. Estrogen deficiency induced by ovariectomy accelerates destructive autoimmune lesions, and these lesions were recovered by estrogen administration in an SS mouse model [45]. It was demonstrated that the dysfunction of regulatory T cells caused by estrogen deficiency may play a crucial role in acceleration of organ-specific autoimmune lesions, and that estrogenic action influences target epithelial cells through Fas-mediated apoptosis [45]. It was also demonstrated that interferon-γ-induced Fas expression on these cells was reduced by the addition of estrogens. Previous studies have shown that physiologic concentration of estrogens augmented the activity of the interferon-γ promotor in mitogen-stimulated murine spleen cells [46], and the administration of exogenous estrogens could induce Fas-mediated apoptosis not only in cultured cells but also *in vivo* [47]. 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 [48,49].

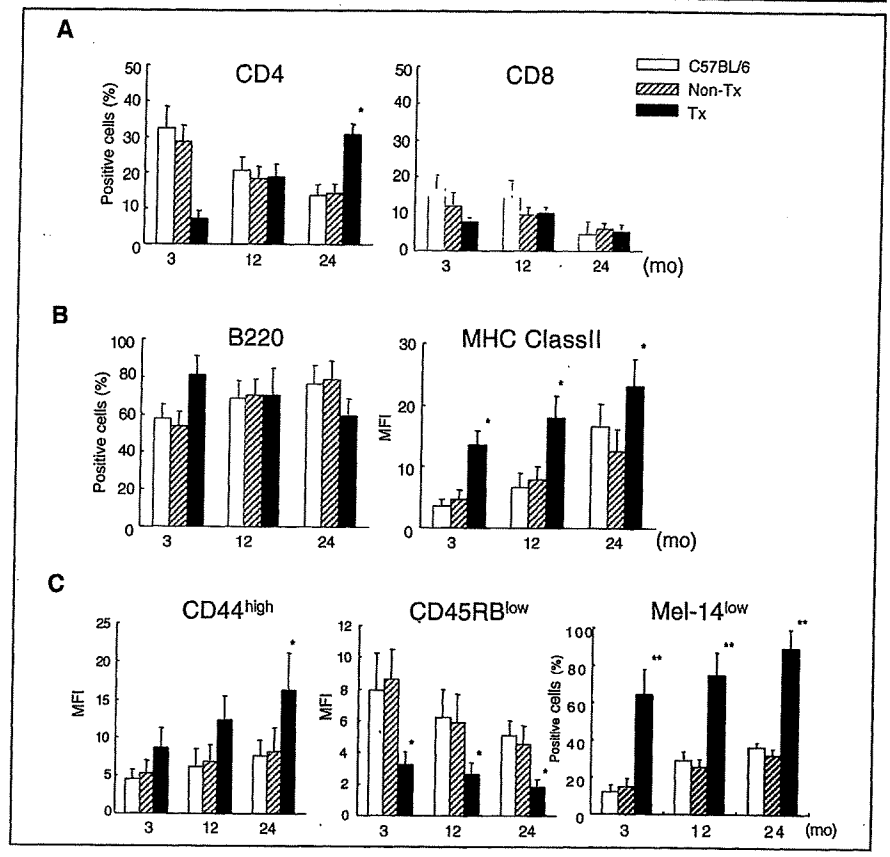
Previous studies concerning gender differences in autoimmunity have suggested that estrogen influences the cytokine production of effector cells and autoantibody production [50,51]. The distinct immune environments in males and females underlie many of the gender-related differences in autoimmunity. 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 [52]. Estrogen withdrawal after menopause leads to an increase in the production of cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-1, interleukin-6, and tumor necrosis factor-α [53]. Although many studies have described the effects of estrogen on cytokine production in effector cells, much less is known about the effect of estrogen deficiency in target organs of postmenopausal women. Recent data demonstrated significant apoptosis associated with α-fodrin cleavage in the salivary gland cells of estrogen-deficient healthy C56BL/6(B6) mice [54••]. Interestingly, inflammatory lesions developed exclusively in the salivary and lacrimal gland after the adoptive transfer with α-fodrin-reactive T cells in both ovariectomized B6 and ovariectomized SCID mice. It has been suggested that α-fodrin cleavage triggered by estrogen deficiency plays an important role in the development of autoimmune exocrinopathy in SS. In *in vitro* studies using primary cultured MSG and human salivary gland cells (HSG), a cleavage product of 120-kD α-fodrin was detected in cells that had undergone tamoxifen (Tam)-induced apoptosis, not in other type of cells including MCF-7 [54••]. Because 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. A recent report by Morkuniene *et al.* [55] has shown that 17β-estradiol prevents calcium-induced release of cytochrome c from heart mitochondria. 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, the promoter activity of caspase 1 was significantly increased when HSG cells transfected with the promoter-caspase 1 gene were stimulated with Tam.

## Conclusion

A cleavage product of 120-kDa α-fodrin was identified as an important organ-specific autoantigen in human SS. The data discussed in this review are strongly suggestive of essential roles of caspase cascade for α-fodrin autoantigen cleavage leading to tissue destruction in autoimmune exocrinopathy in SS. α-Fodrin cleavage by caspases can potentially lead to cytoskeletal rearrangement, and it is of interest to point out that α-fodrin binds to ankylin, which contains a cell death domain [56]. It has been shown that cleavage products of α-fodrin inhibit ATP-dependent glutamate and γ-aminobutyric acid accumulation into synaptic vesicles [57], assuming that a cleavage product of 120 kDa α-fodrin could be a novel component of an unknown immunoregulatory networks such as cytolinker proteins [58]. *In vitro* T-cell apoptosis assay indicated that FasL-mediated AICD is down-regulated by autoantigen stimulation in spleen cells from murine SS. The processing of sFasL occurs in autoantigen-specific $CD4^+$ T cells *in vivo*, and a significant increase in expressions of metalloproteinase-9 mRNA was observed in spleen cells from mouse model. These data indicate that the increased generation of sFasL inhibits the normal AICD process, leading to the proliferation of effector $CD4^+$ T cell (Fig. 1). Moreover, antiestrogenic actions have a potent effect on the proteolysis of α-fodrin autoantigen through up-regulation of caspase 1 activity. It has been strongly suggested that α-fodrin fragments induced by estrogen deficiency may

**Figure 1. An organ-specific autoantigen may play an important role on down-modulation of AICD**

A cleavage product of 120-kD α-fodrin in the target cells could be induced by estrogen deficiency during apoptosis through caspase activation, in particular caspase 1. Activation-induced cell death (AICD) results from the interaction between Fas and FasL, and activated T cells expressing both Fas and FasL are usually killed either by themselves or by interacting with each other. FasL undergo matrix metalloproteinase (MMP)–mediated proteolytic processing in their extracellular domains, resulting in the release of soluble FasL (sFasL). FasL-mediated AICD is down-regulated by autoantigen stimulation, indicating that the increased generation of soluble FasL inhibits the normal AICD process, leading to the proliferation of autoreactive $CD4^+$ T cells. A defect in AICD may result in the development of autoimmune diseases.




play an important role in the development of autoimmune lesions in SS. Molecular mechanisms responsible for tissue-specific apoptosis induced by estrogen deficiency are being further investigated.

## References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • Of special interest
- •• Of outstanding interest


1 Miller JF: Self-nonself discrimination and tolerance in T and B lymphocytes. Immunol Res 1993, 12:115–130.

2 Yan J, Mamula MJ: Autoreactive T cells revealed in the normal repertoire: escape from negative selection and peripheral tolerance. J Immunol 2002, 168:3188–3194.

3 Fox RI, Stern M, Michelson P: Update in Sjögren's syndrome. Curr Opin Rheumatol 2000, 12:391–398.

4 Manoussakis MN, Moutsopoulos HM: Sjögren's syndrome: current concepts. Adv Intern Med 2001, 47:191–217.

5 Chan EK, Hamel JC, Buyon JP, et al.: Molecular definition and sequence motifs of the 52-kD component of human SS-A/Ro autoantigen. J Clin Invest 1991, 87:68–76.

6 Bieganowska KD, Ausubel LJ, Modabber Y, et al.: Direct ex vivo analysis of activated, Fas-sensitive autoreactive T cells in human autoimmune disease. J Exp Med 1997, 185:1585–1594.

7 Casciola-Rosen L, Rosen A, Petri M, et al.: Surface blebs on apoptotic cells are sites of enhanced procoagulant activity implications for coagulation events and antigenic spread in systemic lupus erythematosus. Proc Natl Acad Sci USA 1996, 93:1624–1629.

8 Miranda-Carus ME, Askanase AD, Clancy RM, et al.: Anti-SSA/Ro and anti-SSB/La autoantibodies bind the surface of apoptotic fetal cardiocytes and promote secretion of TNF-alpha by macrophages. J Immunol 2000, 165:5345–5351.

9 Utz PJ, Hottelet M, Schur PH, et al.: Proteins phosphorylated during stress-induced apoptosis are common targets for autoantibody production in patients with systemic lupus erythematosus. J Exp Med 1997, 185:843–854.

10 Humpherys-Beher MG, Peck AB, Dang H, et al.: The role of apoptosis in the initiation of the autoimmune response in Sjögren's syndrome. Clin Exp Immunol 1999, 116:383–387.

11 Ito M, Terasaki S, Itoh J, et al.: Rheumatic disease in an MRL strain of mice with a deficit in functional Fas ligand. Arthritis Rheum 1997, 40:1054–1063.

12 Nishio A, Katakai T, Oshima C, et al.: A possible involvement of Fas-Fas ligand signaling in the pathogenesis of murine autoimmune gastritis. Gastroenterology 1996, 111:956–967.

13 Giordano C, Stassi G, De Maria R, et al.: Potential involvement of Fas and its ligand in the pathogenesis of Hashimoto's thyroiditis. Science 1997, 275:960–963.

14 Apostolou I, Hao Z, Rajewsky K: et al.: Effective destruction of Fas-deficient insulin-producing β cells in type I diabetes. J Exp Med 2003, 198:1103–1106.

15 • Silva DG, Socha L, Charlton B, et al.: Autoimmune diabetes in the NOD mouse: an essential role of Fas-FasL signaling in β cell apoptosis. Ann NY Acad Sci 2003, 1005:161–165.
The study demonstrated that, in the presence of an inflammatory infiltrate, FasL-expressing β cells are exquisitely sensitive to Fas-mediated apoptosis and that this can be blocked by preventing FasL-Fas interaction.

16 Kong L, Ogawa N, Nakabayashi T, et al.: Fas and Fas ligand expression in salivary glands of patients with primary Sjögren syndrome. Arthritis Rheum 1997, 40:87–97.

17 Haneji N, Nakamura T, Takio K, et al.: Identification of α-fodrin as a candidate autoantigen in primary Sjögren's syndrome. Science 1997, 276:604–607.

18 Leto TL, Pleasic S, Forget BG, et al.: Characterization of the calmodulin-binding site of nonerythroid α-spectrin. J Biol Chem 1989, 264:5826–5830.

19 Martin SD, Finucane DM, Amarante-Mendes GP, et al.: Phosphatidylserine externalization during CD95-induced apoptosis of cells and cytoplasts requires ICE/CED-3 protease activity. J Biol Chem 1996, 271:28753–28753.

20 Vanags DM, Pörn-Ares I, Coppolaa S, et al.: Protease involvement in fodrin cleavage and phosphatidylserine exposure in apoptosis. J Biol Chem 1996, 271:31075–31085.

21 Janicke RU, 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.

22 Nagaraju K, Cox A, Caciola-Rosen L, et al.: Novel fragments of the Sjögren's syndrome autoantigens α-fodrin and type 3 muscarinic acetylcholine receptor generated during cytotoxic lymphocyte granule- induced cell death. Arthritis Rheum 2001, 44:2376–2386.

23 Saegusa K, Ishimaru N, Yanagi K, et al.: Prevention and induction of autoimmune exocrinopathy is dependent on pathogenic autoantigen cleavage in murine Sjögren's syndrome. J Immunol 2002, 169:1050–1057.

24 Hahn S, Gehri R, Erb P: Mechanism and biological significance of CD4- mediated cytotoxicity. Immunol Rev 1995, 146:57–79.

25 Rudel T, Bokoch GM: Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. Science 1997, 276:1571–1574.

26 Huang S, Jiang Y, Li Z, et al.: Apoptosis signaling pathway in T cells is composed of ICE/Ced-3 family proteases and MAP kinase kinase 6β. Immunity 1997, 6:739.

27 Tewan M, Quan LT, O'Rouker K, et al.: Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly (ADP-ribose) polymerase. Cell 1995, 81:801–809.

28 Casciola-Rosen L, Nicholson DW, Chong T, et al.: Apopain/CPP32 cleaves proteins that are essential for cellular repair: a fundamental principle of apoptotic death. J Exp Med 1996, 183:1957–1964.

29 Brunner T, Mogil RJ, LaFace D, et al.: Cell-autonomous Fas(CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. Nature 1995, 373:441–444.

30 Thompson CB: Apoptosis in the pathogenesis and treatment of disease. Science 1995, 267:1456–1462.

31 •• Xu L, Zhang L, Kang HK, et al: Human lupus T cells resist inactivation and escape death by upregulating COX-2. Nat Med [serial online] 2004, doi: 10.1038/nm1005.

Recent article demonstrates that activated T cells of lupus patients resist anergy and apoptosis by markedly upregulating and sustaining cyclooxygenase-2 (COX-2) expression. Studies with COX-2 inhibitors and Cox-2-deficient mice confirmed that this COX-2/FLIP antiapoptosis program is used selectively by anergy-resistant lupus T cells. It was also found that only COX-2 inhibitors were able to suppress the production of pathogenic autoantibodies to DNA by causing autoimmune T cell apoptosis.

32 Drappa J, Brot N, Elkon KB: The Fas protein is expressed at high levels on $CD4^+CD8^+$ thymocytes and activated mature lymphocytes in normal mice but not in the lupus-prone strain, MRL-lpr/lpr. Proc Natl Acad Sci USA 1993, 90:10340–10344.

33 Ishimaru N, Yanagi K, Ogawa K, et al.: Possible role of organ-specific autoantigen for Fas ligand-mediated activation-induced cell death (AICD) in murine Sjögren's syndrome. J Immunol 2001, 167:6031–6037.

34 Kabelitz D, Oberg H-H, Pohl T, et al.: Antigen-induced death of mature T lymphocytes: analysis by flow cytometry. Immunol Rev 1994, 142:157–174.

35 Pelfrey CM, Tranquill LR, Boehme SA, et al.: Two mechanisms of antigen-specific apoptosis of myelin basic protein (MBP)-specific T lymphocytes derived from multiple sclerosis patients and normal individuals. J Immunol 1995, 154:6191–6202.

36 Fisher GH, Rosenberg FJ, Strauss SE, et al.: Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. Cell 1995, 81:935–946.

37 Finnegan A, Mikecz K, Tao P, et al.: Proteoglycan (aggrecan)-induced arthritis in BALB/c mice is a Th1-type disease regulated by Th2 cytokines. J Immunol 1999, 163:5383–5390.

38 Lahita RG: Sex steroids and the rheumatic diseases. Arthritis Rheum 1985, 28:121–126.

39 Bateman A, Singh A, Kral T: The immune-hypothalamic-pituitary-adrenal axis. Endocrinol Rev 1989, 10:92–112.

40 Lahita RG, Bradlow L, Fishman J, et al.: Estrogen metabolism in systemic lupus erythematosus: patients and family members. Arthritis Rheum 1982, 25:843–846.

41 Daniels T, Whitcher JP: Association of patients of labial salivary gland inflammation with keratoconjunctivitis sicca. Analysis of 618 patients with suspected Sjögren's syndrome. Arthritis Rheum 1994, 37:869–877.

42 Ishimi Y, Miyaura C, Ohmura M, et al.: Selective effects of genistein, a soybean isoflavone, on B-lymphopoiesis and bone loss caused by estrogen deficiency. Endocrinology 1999, 140:1893–1900.

43 Masuzawa T, Miyaura C, Onoe Y, et al.: Estrogen deficiency stimulates B lymphopoiesis in mouse bone marrow. J Clin Invest 1994, 94:1090–1097.

44 Mor G, Sapi E, Abrahams VM, et al.: Interaction of the estrogen receptors with the Fas ligand promotor in human monocytes. J Immunol 2003, 170:114–122.

45 Ishimaru N, Saegusa K, Yanagi K, et al.: Estrogen deficiency accelerates autoimmune exocrinopathy in murine Sjögren's syndrome through Fas-mediated apoptosis. Am J Pathol 1999, 155:173–181.

46 Fox HS, Bond BL, Parslow TG: Estrogen regulates the IFN-γ promoter. J Immunol 1991, 146:4362–4367.

47 Takabayashi H, Oida H, Fujisawa K, et al.: Hormone-induced apoptosis by Fas-nuclear receptor fusion protein: novel biological tools for controlling apoptosis in vivo. Cancer Res 1996, 56:4164–4170.

48 Spyridopoulos I, Sullivan A, Kearney M, et al.: Estrogen-receptor-mediated inhibition of human endothelial cell apoptosis. Estradiol as a survival factor. Circulation 1997, 95:1505–1514.

49 Pelzer T, Schumann M, Neumann M, et al.: 17β-Estradiol prevents programmed cell death in cardiac myocytes. Biochem Biophys Res Commun 2000, 268:192–200.

50 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.

51 Ansar-Ahmed S, Dauphinee M, Montoya A, et al.: Estrogen induces normal murine $CD5^+$ B cells to produce autoantibodies. J Immunol 1989, 142:2647–2653.

52 Whitacre CC, Reingold SC, O'Looney PA: A gender gap in autoimmunity. Science 1999, 283:1277–1278.

53 Kimble RB, Srivastava S, Ross FP, et al.: 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.

54 •• Ishimaru N, Arakaki R, Watanabe M, et al.: Development of autoimmune exocrinopathy resembling Sjögren's syndrome in estrogen-deficient mice of healthy background. Am J Pathol 2003, 163:1481–1490.

This study evaluated the effects on autoantigen cleavage in estrogen-deficient healthy C57BL/6 (B6) mice treated with an ovariectomy. A significant increase in apoptotic epithelial salivary gland cells was associated with α-fodrin cleavage. A cleavage product of 120-kDa α-fodrin was detected in salivary gland cells that had undergone tamoxifen-induced apoptosis through caspase activation, especially caspase-1. Adoptive transfer of α-fodrin-reactive T cells into ovariectomized B6 mice resulted in the development of autoimmune exocrinopathy quite similar to Sjögren syndrome.

55 Morkuniene R, Jekabsone A, Borutaite V: Estrogens prevents calcium-induced release of cytochrome c from heart mitochondria. FEBS Lett 2002, 521:53–56.

56 Feinstein E, Kimchi A, Wallach D, et al.: The death domain: a module shared by proteins with diverse cellular function. Trends Biochem Sci 1995, 20:342–344.

57 Ozkan ED, Lee FS, Ueda T: A protein factor that inhibits ATP-dependent glutamate and γ-aminobutyric acid accumulation into synaptic vesicles: purification and initial characterization. Proc Natl Acad Sci USA 1997, 94:4137–4142.

58 Brown MJ, Hallam JA, Yamada KM, et al.: Integration of human T lymphocyte cytoskeleton by cytolinker protein. J Immunol 2001, 167:641–645.

American Journal of Pathology, Vol. 165, No. 1, July 2004


# Molecular Analysis of the Human Autoantibody Response to α-Fodrin in Sjögren's Syndrome Reveals Novel Apoptosis-Induced Specificity

Toshiaki Maruyama,* Ichiro Saito,† Yoshio Hayashi,‡ Elizabeth Kompfner,* Robert I. Fox,§ Dennis R. Burton,* and Henrik J. Ditzel*¶‖

*From the Department of Immunology,* The Scripps Research Institute, La Jolla, California; the Allergy and Rheumatology Clinic,§ The Scripps Memorial Hospital, La Jolla, California; the Department of Pathology,† Tsurumi University School of Dental Medicine, Yokohama, Japan; the Department of Pathology,‡ Tokushima University School of Dentistry, Tokushima, Japan; Center of Medical Biotechnology,¶ Institute of Medical Biology, University of Southern Denmark, Odense, Denmark; and Department of Internal Medicine C,‖ Odense University Hospital, Odense, Denmark*

**Lymphocyte infiltration of salivary and lacrimal glands leading to diminished secretion and gland destruction as a result of apoptosis is thought to be pivotal in the pathogenesis of Sjögren's syndrome (SS). The cytoskeletal protein α-fodrin is cleaved during this apoptotic process, and a strong antibody (Ab) response is elicited to a 120-kd fragment of cleaved α-fodrin in the majority of SS patients, but generally not in other diseases in which apoptosis also occurs. Little is known about the anti-α-fodrin autoantibody response on a molecular level. To address this issue, IgG phage display libraries were generated from the bone marrow of two SS donors and a panel of anti-α-fodrin IgGs was isolated by selection on α-fodrin immunoblots. All of the human monoclonal Abs (hmAbs) reacted with a 150-kd fragment and not with the 120-kd fragment or intact α-fodrin, indicating that the epitope recognized became exposed after α-fodrin cleavage. Analysis of a large panel of SS patients (defined by the strict San Diego diagnostic criteria) showed that 25% of SS sera exhibited this 150-kd α-fodrin specificity. The hmAbs stained human cultured salivary acinar cells and the staining was redistributed to surface blebs during apoptosis. They also stained inflamed acinar/ductal epithelial cells in SS salivary tissue biopsies, and only partially co-localized with monoclonal Abs recognizing the full-length α-fodrin. Our study shows that in SS patients, neoepitopes on the 150-kd cleaved product of α-fodrin become exposed to the immune system, frequently eliciting anti-150-kd α-fodrin Abs in addition to the previously reported anti-120-kd Abs. The anti-150-kd α-fodrin hmAbs may serve as valuable reagents for the study of SS pathogenesis and diagnostic analyses of SS salivary gland tissue.** ***(Am J Pathol 2004, 165:53–61)***

Sjögren's syndrome (SS) is the second most common autoimmune rheumatic disease, causing ocular and oral dryness and extraglandular manifestations in three to four million people in the United States alone.[1–3] The disease is characterized by lymphocytic infiltrates and destruction of the salivary and lacrimal glands, and systemic production of characteristic autoantibodies. Xerostomia and keratojunctivitis sicca are the common clinical signs, but the San Diego SS diagnostic criteria also require a positive salivary gland biopsy or the presence of autoantibodies to the ribonucleoprotein SS-A/Ro for diagnosis;[4] these requirements are not included in the European Economic Committee diagnostic criteria.[5,6] The typical histopathological findings of SS salivary and lacrimal gland tissues include glandular attrition in acinar and ductal epithelia concomitant with lymphoplasmacytic infiltration consisting of predominantly $CD4^+$ cells, but also $CD8^+$, B cells, and plasma cells. Several immune and inflammatory effector pathways seem to be implicated in the ongoing pathology of SS, but our understanding of the initiation factors and the precise mechanism of epithelial cell damage and dysfunction remains limited.

Recent studies have indicated a 120-kd fragment of α-fodrin as a potential important autoantigen in the pathogenesis of primary SS in both a mouse model and in humans.[7–11] Fodrin is an abundant component of the membrane cytoskeleton of most eukaryotic cells. It is composed of heterodimers of an α (240 kd) and a β (235 kd) subunit that share homologous internal spectrin repeats, but have distinct amino- and carboxyl-terminal regions. The α-fodrin subunit is an actin-binding protein that may be involved in secretion[12–14] and has been shown in apoptotic cells to be cleaved by calpain,

Supported in part by a research grant from the Sjögren's Syndrome Foundation and the National Institutes of Health (grants AI41590 and HL63651 to H.J.D.).

Accepted for publication March 4, 2004.

Address reprint requests to Henrik J. Ditzel, Center of Medical Biotechnology, University of Southern Denmark, Winsloewparken 25, 3, 5000 Odense C, Denmark. E-mail: hditzel@health.sdu.dk.

caspases, and an unidentified protease present in T-cell granule content.[15–18] Indeed, treatment of mice with caspase inhibitors prevents induction of SS.[19] Autoantibodies to the 120-kd cleavage fragment of α-fodrin have been detected in patients with primary and secondary SS but also in a few systemic lupus erythematosus (SLE) patients without SS.[7,9,20–22] Different diagnostic criteria for SS have been used in the various studies and differences in the specificity of 120-kd α-fodrin for SS have been observed, which has rendered the importance of 120-kd α-fodrin as a diagnostic marker controversial.

Here, we have further evaluated the incidence and specificity of anti-α-fodrin Ab response in American SS patients and found a correlation between anti-α-fodrin Ab and SS, but a lower prevalence of anti-120-kd α-fodrin Abs in American *versus* Japanese SS patients. We also found that ~25% of SS sera contained Abs against the 150-kd cleavage fragment of α-fodrin. To examine these Abs at a molecular level, we cloned and characterized a panel of hmAbs from SS patients using phage display technology that specifically recognized the 150-kd α-fodrin neoepitope. The anti-150-kd hmAbs were shown to detect 150-kd α-fodrin in apoptotic acinar and ductal salivary gland cells in cell culture, and in SS salivary gland tissue sections, indicating that the hmAbs may be useful diagnostic reagents in SS pathology.

## Materials and Methods

### Patients

Sera were obtained from 60 SS patients (42 American and 18 Japanese) who fulfilled the San Diego criteria for the diagnosis of SS;[23] 12 rheumatoid arthritis (RA) patients; 12 SLE patients, diagnosed based on American College of Rheumatology criteria; and 10 healthy individuals. Bone marrow from two Caucasian American patients with secondary SS (designated SS23 and SS30) were obtained for Ab library construction.

### Western Blot Analysis

α-Fodrin was purified from mouse brain tissue using the method of Cheney and colleagues[24] yielding >95% purity. Mouse α-fodrin exhibits 94% amino acid sequence identity to human α-fodrin. Coomassie staining of mouse brain α-fodrin separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% Tris-HCl gel (Bio-Rad, Hercules, CA) showed an intense band at 240 kd corresponding to intact α-fodrin, but also weaker bands at 180, 150, 120, 80, 50, and 30 kd that corresponded to cleaved α-fodrin because of low levels of constitutive apoptosis, as previously reported.[14,17,24,25] Mouse brain α-fodrin separated by SDS-PAGE was also electroblotted onto polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, MA), the membrane was blocked with 5% nonfat dry milk (Bio-Rad) in phosphate-buffered saline (PBS), pH 7.0, for 30 minutes, and incubated with serum (diluted 1:1000 in PBS), human recombinant Fabs (1 to 20 μg/ml) or mouse anti-α-fodrin mAb AA6 (Affiniti, Exeter, UK) for 1 hour on a rotator. mAb AA6 predominantly recognizes the 240-kd intact form of α-fodrin, but also the 120- and 150-kd cleaved form of α-fodrin. The membrane was washed three times (10 minutes/wash) in PBS and bound serum Ab was detected with horseradish peroxidase-conjugated goat Fab anti-human IgG (H+L) Ab (Bio-Rad). A patient serum was used as internal control in each blotting experiment to adjust for band intensity variations between gels. The intensity of the bands was scored (1 to 5) based on quantification by densitometry. Bound human recombinant Fabs were detected with horseradish peroxidase-conjugated goat anti-human IgG $F(ab')_2$ Ab and bound mouse mAb detected with horseradish peroxidase-conjugated goat anti-mouse IgG Ab (both Jackson) diluted in blocking solution and incubated for 1 hour at room temperature. After washing for 45 minutes with PBS, membranes were rinsed briefly in MilliQ water, and bound enzyme-labeled Ab was visualized using chemiluminescent substrate (SuperSignal, WestPico; Pierce, Rockford, IL) according to the manufacturer's instructions and autoradiographic film (Eastman Kodak, Rochester, NY). All incubations were done at room temperature. As controls, all experiments were performed using the anti-Ebola virus Fab ELZ510, the anti-HIV-1 gp120 Fab b12, normal sera or by omitting the primary Ab.

### Analysis of Patient Sera and Human Fabs by Enzyme-Linked Immunosorbent Assay (ELISA)

Mouse brain α-fodrin (2 μg/ml) and ovalbumin (4 μg/ml) (Pierce) were coated onto microtiter wells (Costar, Cambridge, MA) at 4°C overnight. Wells were washed with PBS; blocked with 4% nonfat dry milk in PBS for 30 minutes; and incubated with patient serum (diluted 1:100 and 1:400 in PBS), human Fabs, or mouse anti-α-fodrin mAb AA6 for 1 hour at 37°C. Wells were washed six times with PBS-0.05% Tween and bound Ab was detected with alkaline phosphatase-conjugated goat IgG anti-human IgG $F(ab')_2$ Ab or anti-mouse IgG $F(ab')_2$ Ab (both 1:500 in 1% bovine serum albumin/PBS, Pierce) and visualized with nitrophenol substrate (NPP substrate) (Sigma, St. Louis, MO) by reading absorbance at 405 nm.

### RNA Isolation and Library Construction

RNA was isolated from bone marrow of two American SS patients (designated SS23 and SS30) by a guanidinium isothiocyanate method, as described previously.[26] Serum samples from each donor were drawn concomitantly. After reverse-transcription, the γ1 (Fd region) and κ and λ chains were amplified by polymerase chain reaction and phage-display libraries were constructed in the phage-display vector pComb3, as described previously.[27–29]

### Ab Library Selection

Libraries were selected against α-fodrin blotted membrane. Mouse brain α-fodrin was separated by SDS-

PAGE using a 10% Tris-HCl gel and transferred to polyvinylidene difluoride membranes. After blocking with 5% nonfat dry milk/PBS for 30 minutes, the membrane was incubated with phage ($10^{11}$ pfu) resuspended in PBS containing 1% bovine serum albumin for 2 hours at room temperature. The membrane was washed and the bound phage, enriched for those bearing antigen-binding surface Fabs, were eluted with 0.2 mol/L glycine-HCl buffer, pH 2.2, as previously described.[29–31] The eluted phages were amplified by infection of *Escherichia coli* and superinfection with M13 helper phage. The libraries were panned for four consecutive rounds with increasing washing stringency (2 × 10 minutes for first panning round and 4 × 10 minutes thereafter). Phagemid DNA, isolated after the last round of panning, was digested with *Nhe*I and *Spe*I restriction endonucleases and religated to excise the cpIII gene. The reconstructed phagemid was used to transform XL1-Blue cells (Stratagene, La Jolla, CA) to produce clones secreting soluble Fab fragments. Positive Fab clones were purified from bacterial supernatants by affinity chromatography as previously described.[32]

### *Nucleic Acid Sequencing*

Nucleic acid sequencing was performed on a 373A or 377A automated DNA sequencer (ABI, Foster City, CA) using a *Taq* fluorescent dideoxy terminator cycle sequencing kit (ABI). Sequencing primers were as reported.[33] Comparison to reported Ig germline sequences from GenBank and EMBL was performed using the Genetic Computer Group (GCG) sequence analysis program.

### *Confocal Laser-Scanning Microscopy Analysis of Human Cells and Tissue Biopsies*

Human salivary gland (HSG) cells were grown in minimum essential medium, Eagle's, in Earle's balanced salt solution (EMEM) medium supplemented with 10% fetal calf serum and allowed to adhere to chambered coverslips (Nunc, Kamstrup, Denmark) for 48 hours at 37°C, 5% $CO_2$, to form monolayers. Apoptosis of HSG cells was induced by incubating the cells with 100 ng/ml of tumor necrosis factor-α (Upstate Biotechnology, Lake Placid, NY) and 1 μg/ml of cycloheximide for 3 to 15 hours at 37°C/5% $CO_2$. Untreated cells or those induced to undergo apoptosis were fixed by 96% ice-cold ethanol for 5 minutes at 4°C or by 4% paraformaldehyde for 10 minutes at room temperature. Paraformaldehyde-fixed cells were washed in PBS before being permeabilized in 0.005% saponin for 10 minutes at room temperature. After washing in PBS and blocking with 5% normal goat serum for 1 hour, cells were incubated with Ab. Fresh-frozen tissue was obtained from labial biopsies of patients with active SS and healthy controls. Freshly cut 5-μm sections were dried overnight, fixed by ice-cold 96% ethanol for 5 minutes at 4°C or by acetone for 10 minutes at room temperature, and blocked with 5% normal goat serum. HSG cells and tissue sections were incubated with human Fabs (10 μg/ml in PBS), or mouse anti-α-fodrin mAb AA6 (Affiniti) for 1 hour. In some experiments apoptotic cells were stained with Annexin V-FITC (Pharmingen, La Jolla, CA) for 1 hour before fixation. The slides were washed with PBS and incubated with fluorescein isothiocyanate-labeled $(Fab')_2$ goat anti-human IgG $(Fab')_2$ Ab (Jackson), and Texas Red-labeled goat anti-mouse IgG Ab (Jackson), or propidium iodine (Sigma) for 1 hour. The slides were again washed with PBS for 5 minutes and mounted with Slow Fade in PBS/glycerol (Molecular Probes, Eugene, OR) before analysis using a Zeiss Axiovert S100 TV confocal laser-scanning microscope (Zeiss, New York, NY). All incubations were performed at room temperature unless otherwise indicated. As controls, all experiments were performed using the human Fab b12 to HIV-1 gp120 or by omitting the primary Ab. Adjacent tissue sections were hematoxylin and eosin stained or stained with anti-CD3 (DAKO, Carpinteria, CA), and anti-cytokeratin 18 (CY-90; Sigma-Aldrich) mAbs to determine the cell type present.

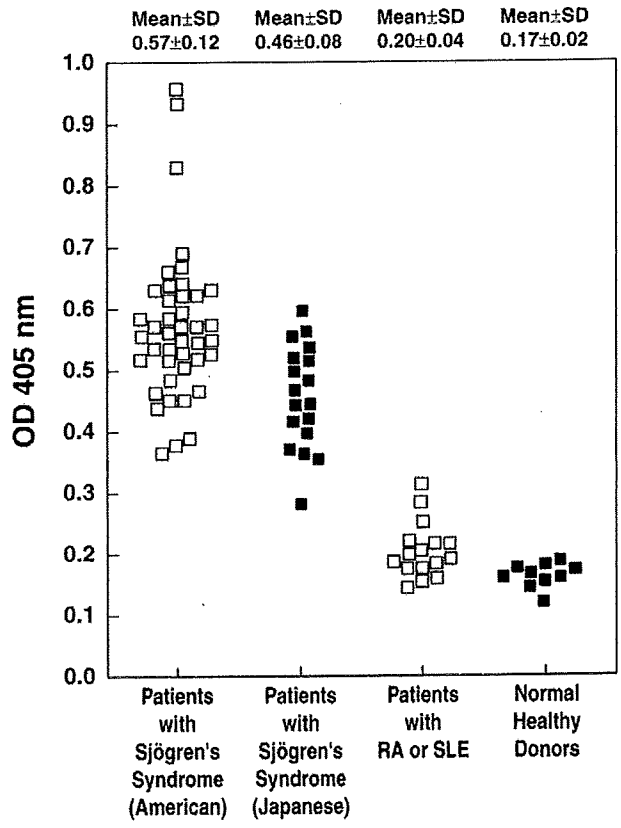



**Figure 1.** Sera from SS patients contain anti-α-fodrin Abs, as measured by ELISA. Sera, diluted 1:400 in PBS, from 42 American SS patients, 17 Japanese SS patients, 16 RA and SLE patients, and 10 healthy individuals were tested for binding to mouse brain α-fodrin by ELISA. Samples with $A_{405}$ values more than twice the mean of the control normals (>0.33) were considered positive.

## *Results*

### *Serological Analysis of α-Fodrin Autoantibodies in SS Patients*

To investigate the specificity and sensitivity of anti-α-fodrin Abs for SS, serum from patients with SS, RA, SLE,

and healthy individuals were tested for binding to mouse brain α-fodrin by ELISA. A secondary Ab capable of detecting both IgG and IgA was used, because anti-α-fodrin Ab of both the IgG and IgA have been suggested to be elevated in SS sera. As shown in Figure 1, elevated α-fodrin Ab levels were observed in both American and Japanese SS patients compared to the RA and SLE patient groups and healthy individuals. Positivity was defined as an $OD_{405}$ value greater than twice the mean of the normal controls (>0.33) at a serum dilution of 1:400. Sera from all of the American SS patients and all but one of the Japanese SS patients were positive for α-fodrin Abs (98%), whereas only 1 of 16 RA/SLE patients (6%), and none of the healthy donors, were positive. The mean level of anti-α-fodrin Ab in both the American ($OD_{405}$ nm, 0.57 ± 0.12) and Japanese SS patients ($OD_{405}$ nm, 0.46 ± 0.08) were significantly higher than healthy controls (0.17 ± 0.02, $P < 0.0001$) or the RA/SLE patients (0.20 ± 0.04, $P < 0.0001$). The mouse anti-α-fodrin mAb AA6 was included in each experiment as a control ($OD_{405}$ nm, 0.8).

The frequency of SS sera with Abs specific for the 120-kd fragment of cleaved α-fodrin was determined by assessing binding to Western blots of mouse brain α-fodrin. Fifteen of 42 American SS sera (36%) (diluted 1:1000) exhibited reactivity against the 120-kd fragment of α-fodrin (Table 1). In addition, 10 sera showed reactivity with a 150-kd fragment of cleaved α-fodrin (24%), while 7 showed reactivity with a 180-kd fragment and other cleaved products of α-fodrin. As shown in Table 1, some patient sera reacted with multiple α-fodrin fragments, whereas the Western blot for other sera revealed reactivity with only one of the fragments. Overall, 22 American SS sera (52%) reacted with at least one form of cleaved α-fodrin. When the 17 Japanese SS sera were tested by Western blotting, the prevalence of anti-α-fodrin Ab reactivity was found to be significantly higher than in the American SS sera (12 of 17 positive, 70.6%; $P < 0.01$). All of the α-fodrin-reactive sera from the Japanese patients recognized 120-kd, but five also bound to the 150-kd α-fodrin (29.4%) (Table 1). Serum of 8 RA patients, 8 SLE patients, and 10 healthy individuals was also tested by Western blot analysis. Only one SLE serum was found to react with the 120-kd fragments of α-fodrin and none reacted with the 150-kd fragments of α-fodrin.

**Table 1.** Binding of American (ASSP) and Japanese (JSSP) SS Patient Sera to Cleaved Mouse Brain α-Fodrin by Western Blot Analysis

| Patient | Intensity* of bands | Fragment size (kd) |
|---|---|---|
| ASSP1 | – | |
| ASSP2 | – | |
| ASSP3 | 1+ | 150 |
| ASSP4 | – | |
| ASSP5 | – | |
| ASSP6 | 1+ | 120 |
| ASSP7 | 3+ | 180 |
| ASSP8 | 3+ | 120, 150, 50 |
| ASSP9 | 2+ | 120 |
| ASSP10 | – | |
| ASSP11 | – | |
| ASSP12 | 1+ | 150, 80, 30 |
| ASSP13 | – | |
| ASSP14 | – | |
| ASSP15 | – | |
| ASSP16 | – | |
| ASSP17 | – | |
| ASSP18 | – | |
| ASSP19 | 1+ | 120, 150 |
| ASSP20 | 1+ | 120, 150 |
| ASSP21 | – | |
| ASSP22 | 2+ | 150, 180 |
| ASSP23 | 4+ | 120, 150 |
| ASSP24 | – | |
| ASSP25 | 2+ | 150 |
| ASSP26 | – | |
| ASSP27 | 2+ | 150 |
| ASSP28 | 3+ | 120, 180 |
| ASSP29 | 2+ | 120, 180 |
| ASSP30 | 5+ | 150 |
| ASSP31 | 3+ | 120 |
| ASSP32 | – | |
| ASSP33 | – | |
| ASSP34 | 3+ | 120 |
| ASSP35 | 2+ | 120, 180, 50 |
| ASSP36 | 1+ | 120 |
| ASSP37 | – | |
| ASSP38 | – | |
| ASSP39 | 1+ | 120, 180, 50 |
| ASSP40 | – | |
| ASSP41 | 2+ | 120, 180, 80 |
| ASSP42 | 2+ | 120, 50 |
| JSSP1 | 3+ | 120 |
| JSSP2 | 2+ | 120 |
| JSSP3 | 2+ | 120, 150 |
| JSSP4 | 2+ | 120 |
| JSSP5 | 2+ | 120, 150 |
| JSSP6 | 1+ | 120 |
| JSSP7 | 1+ | 120, 150 |
| JSSP8 | 1+ | 120 |
| JSSP9 | 1+ | 120 |
| JSSP10 | 1+ | 120, 150 |
| JSSP11 | 1+ | 120 |
| JSSP12 | 1+ | 120, 150 |
| JSSP13 | – | |
| JSSP14 | – | |
| JSSP15 | – | |
| JSSP16 | – | |
| JSSP17 | – | |

*The intensity was evaluated at a scale 1 to 5; 5 being the most intense.

## *Isolation of Human IgG mAbs Against the 150-kd Form of Cleaved α-Fodrin from SS Patients*

To characterize the anti-150-kd α-fodrin Abs response at a molecular level, $IgG_1$ κ/λ Ab phage display libraries were generated from two patients (SS23 and SS30), whose sera predominantly recognized the 150-kd cleavage fragment of α-fodrin and also reacted with extract of human SS salivary gland tissue. As starting material for the Ab library construction, bone marrow was obtained from patients SS23 and SS30. Bone marrow has been shown to be a major repository for the plasma cells that produce the Abs found in the circulation. The Ab libraries from patients SS23 (≈6 × $10^6$ members) and SS30 (≈8 × $10^6$ members) were panned against mouse brain α-fodrin consisting of mostly intact α-fodrin, but also a small amount of apoptotic cleaved α-fodrin. The α-fodrin preparation was either separated by SDS-PAGE and

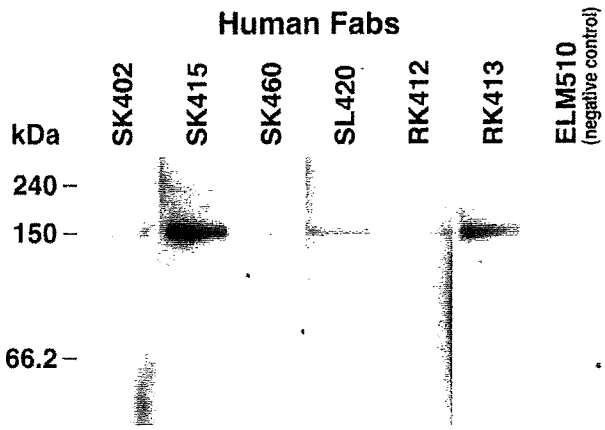



**Figure 2.** Binding of human monoclonal IgG Fabs to purified mouse α-fodrin brain extract by Western blot analysis. All of the Fabs specifically bound to the 150-kd fragment of cleaved α-fodrin and did not react with 240-kd intact α-fodrin. The human monoclonal anti-Ebola virus Fab ELZ510 was used as a negative control.

transferred to a polyvinylidene difluoride membrane or coated on microtiter wells. After four rounds of biopanning against α-fodrin blotted on a polyvinylidene difluoride membrane, a significant increase in eluted phage was observed, indicating enrichment for antigen-binding Fab-phages. Individual clones, expressed as soluble Fabs by excising the gene III from the pcomb3 phagemid DNA from the last round of selection, were tested for binding to α-fodrin by Western blotting. This analysis yielded 19 Fabs that exhibited strong binding to the 150-kd fragment of cleaved α-fodrin and no binding to intact α-fodrin (240 kd), although present in significant excess, or the 120-kd cleaved form of α-fodrin (Figure 2). The Fabs also failed to react with Western blots of HeLa lysate, which contained intact α-fodrin, but not 120- or 150-kd α-fodrin, as determined by staining with mouse mAb AA6. Sequencing the DNA encoding the heavy chain variable region of the 19 Fabs revealed that 4 Fabs isolated from patient SS23 (SK402, SK415, SK460, and SL420) and 2 Fabs isolated from patient SS30 (RK412 and RK413) were unique, whereas the remaining sequences were repeats of the six sequences (Table 2).

## *The IgG-Derived Anti-150-kd α-Fodrin Fabs Are Derived as a Result of an Affinity-Matured Antigen-Driven Antibody Response*

Next, the variable heavy and light chain genes of the IgG-derived anti-150-kd α-fodrin Fab fragments were compared with the closest germline sequences in the GenBank database (Table 2). The Ab heavy chain is the major contributor to antigen-binding in many instances and so detailed analysis was focused on this chain. All of the variable heavy chain region genes of the anti-150-kd α-fodrin IgG Fabs were highly mutated, and exhibited a high replacement (R) to silent (S) mutation ratio (R/S ratio) for the complementarity determining regions (CDRs) (CDR1 and CDR2) compared to the framework regions (FR) (FR1, FR2, and FR3), characteristic of an affinity-matured antigen-driven Ab response. No particular restriction in the VH or JH gene usage of the anti-150-kd α-fodrin IgG Fabs was observed (Table 2), neither did the analysis reveal any restriction in the germline usage within the context of the VH families used. Unequivocal identification of the closest germline D segment proved impossible because of significant somatic modification.

## *Epitope Characterization*

To pinpoint the epitopes recognized by the anti-150-kd α-fodrin human Fabs, three polypeptides spanning α-fodrin were coated on ELISA wells and tested for reactivity with the Fabs. The polypeptides included JS-1 (1 to 1784 bp), 2.7A (2258 to 4884 bp), and 3′DA (3963 to 7083 bp). None of the tested Fabs recognized any of the peptides, suggesting that the Fabs either recognized regions or partial conformational epitopes of α-fodrin not represented by the peptides.

## *Subcellular Distribution of 150-kd α-Fodrin in HSG Cells*

To determine the subcellular distribution of the 150-kd fragment of cleaved α-fodrin, HSG cells were stained with selected human Fabs and examined by laser-scanning confocal microscopy. As shown in Figure 3, Fab SK415 and SK460 exhibited cytoplasmic staining, whereas no staining was observed with a control Fab. Similar cytoplasmic staining was also observed with mouse mAb AA6 recognizing both intact and cleaved α-fodrin (Figure 3I). As previously reported, the cytoplasmic staining of the mouse mAb AA6 was particularly intense, corresponding to the plasma membrane, but this was not observed with the human anti-150-kd α-fodrin Fabs. The fixation method of the HSG cells (ethanol or paraformaldehyde/saponin) did not seem to influence the staining patterns of Fab SK415 and mAb AA6.

**Table 2.** Deduced Amino Acid Sequences of the Heavy Chain CDR3 Region and Adjacent Framework Regions of Anti-150 kd, α-Fodrin IgGs

| Fab | VH/germline | FR3 | CDR3 | FR4 | JH |
|---|---|---|---|---|---|
| SK402 (3) | VH1–8 | YYCAR | EGWPPTNDY | WGQ | JH4 |
| SK415 (1) | VH3–21 | YFCVR | DLCGGRDS | WGQ | JH5 |
| SK460 (2) | VH3–33 | YLCAR | EAWHDIGEYDGRRTLGSVPSLDL | WGQ | JH5 |
| SL420 (1) | VH3–21 | YYCAR | DGDGYRDY | WGQ | JH4 |
| RK412 (8) | VH1–69 | YYCAR | GFGGEDAYYDNFGYYASTEF | WGL | JH3 |
| RK413 (4) | VH4–4 | YYCAR | WGPRDLSGRSGGFDP | WGP | JH4 |

Number in parentheses denotes the number of Fabs that had the same heavy-chain CDR3 sequence. The closest germline gene, VH and JH families for each clone are also shown.

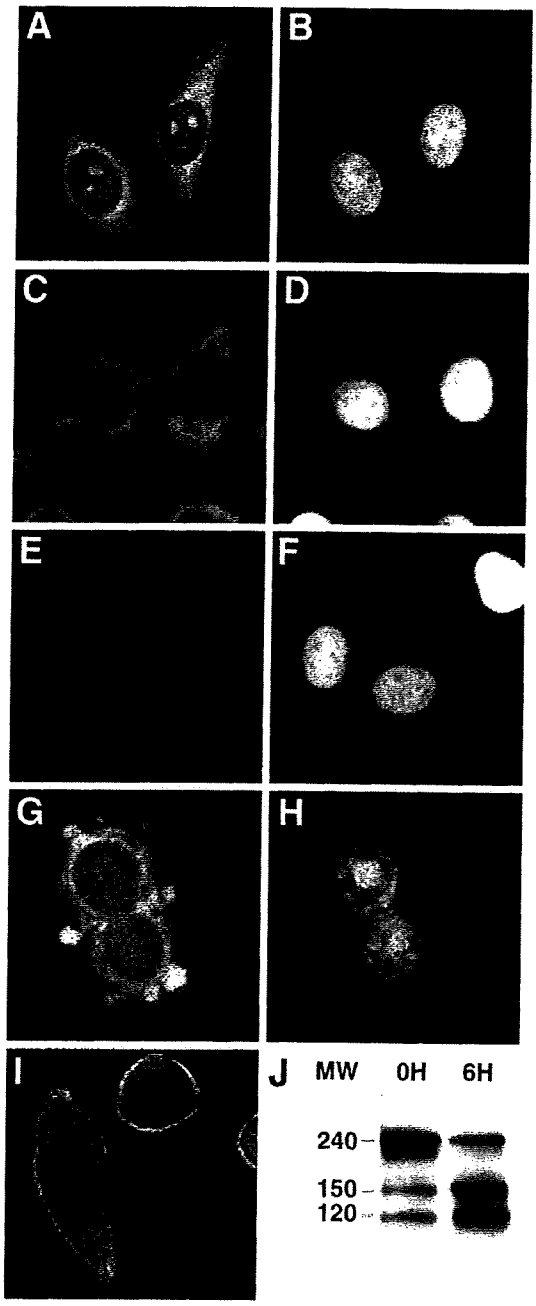



**Figure 3.** Subcellular distribution of intact and cleaved α-fodrin in human salivary cells. Ethanol-fixed human salivary HSG cells were stained with the human anti-150-kd α-fodrin Fabs SK415 (**A**) and SK460 (**C**), a human anti-HIV-1 gp120 Fab, b12 (**E**, negative control), and the mouse anti-α-fodrin 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 iodine (**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 exposed 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 iodine (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.

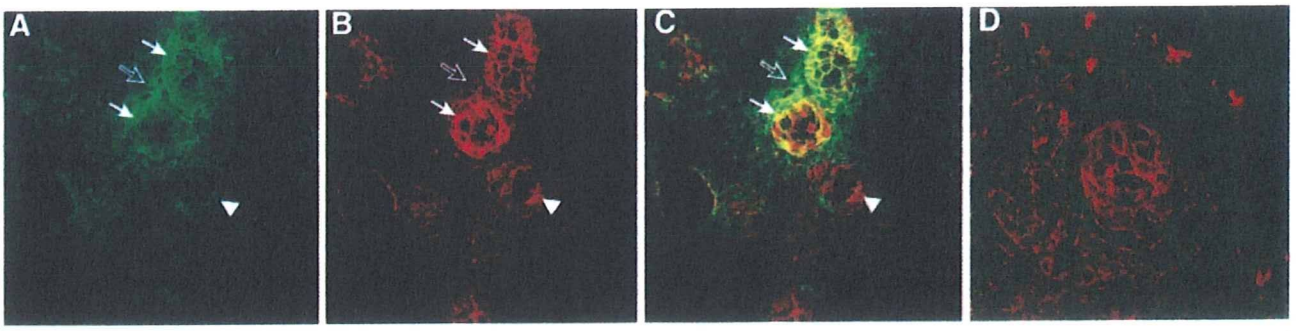



**Figure 4.** Distribution of cleaved and intact α-fodrin in salivary gland tissue of a SS patient with active disease. Tissue sections from salivary gland biopsies of patients with SS were stained with human anti-150-kd α-fodrin Fab SK415 (**A**, green) and mouse anti-α-fodrin mAb AA6 (**B**, red) and 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 though 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 that 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, Vestrheim 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, Konttinen 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, Westerdaal 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, Kukulies 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-a-fodrin antibodies: not specific nor sensitive for Sjögren's syndrome. Arthritis Rheum 1999, 42(Abstract 428 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 Sjogren's syndrome. Arthritis Rheum 1997, 40:87–97
39. Dutta S, Chiu YC, Probert AW, Wang KK: Selective release of calpain produced alphaII-spectrin (alpha-fodrin) breakdown products by acute neuronal cell death. Biol Chem 2002, 383:785–791

Oral Science International, November 2004, p.55-64



Review Article

# Crucial Role of Tissue-specific Apoptosis on the Development of Primary Sjögren's Syndrome

Yoshio Hayashi, Rieko Arakaki and Naozumi Ishimaru

*Department of Oral Molecular Pathology,*
*Institute of Health Bioscience,*
*The University of Tokushima Graduate School*
*(Chief: Professor Yoshio Hayashi)*

**Abstract**: Primary Sjögren's syndrome is an autoimmune disorder characterized by lymphocytic infiltrates and destruction of the salivary and lacrimal glands, and systemic production of autoantibodies to the ribonucleoprotein (RNP) particles SS-A/Ro and SS-B/La, leading to clinical symptoms of dryness of the mouth and eyes (sicca syndrome). Autoreactive T cells bearing the CD4 molecule may recognize an unknown self antigen, triggering autoimmunity in the salivary and lacrimal glands. Although several candidate autoantigens including $\alpha$-fodrin have been reported in Sjögren's syndrome, the pathogenic roles of the autoantigens in initiation and progression of SS are still unclear. It is possible that individual T cells activated by an appropriate self antigen can proliferate and form a restricted clone. Recent evidence suggests that the apoptotic pathway plays a central role in making T cells tolerant to tissue-specific self antigen, and may drive the autoimmune phenomenon. We recently reported that tissue-specific apoptosis in estrogen-deficient mice may contribute to autoantigen cleavage, leading to the development of autoimmune exocrinopathy. The studies reviewed imply that tissue-specific apoptosis and caspase-mediated $\alpha$-fodrin proteolysis are involved in the progression of autoimmune lesions in Sjögren's syndrome. Moreover, Fas ligand (FasL) and its receptor Fas are essential in the homeostasis of the peripheral immune system. It is considered that a defect in activation-induced cell death (AICD) of effector T cells may result in the development of autoimmune exocrinopathy in Sjögren's syndrome.

**Key words**: Sjögren's syndrome, apoptosis, autoantigen, activation-induced cell death (AICD), estrogen deficiency

## Introduction

Organ-specific autoimmune diseases are characterized by tissue destruction and functional decline due to autoreactive T cells that escape self-tolerance[1,2]. Sjögren's syndrome (SS) is a T cell-mediated autoimmune disorder characterized by lymphocytic infiltrates and destruction of the salivary and lacrimal glands, and systemic production of autoantibodies to the ribonucleoprotein (RNP) particles SS-A/Ro and SS-B/La[3,4]. Autoreactive T cells bearing the CD4 molecule may recognize an unknown autoantigen triggering autoimmunity in the salivary and lacrimal glands, leading to clinical symptoms of dryness of the mouth and eyes (sicca syndrome)[5,6]. Although it has been argued that T cells play an important role in the development of organ-specific autoimmune disease, it is

Received 9/29/04; accepted 10/22/04.
**Grant support**: Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan
**Requests for reprints**: Yoshio Hayashi, Department of Oral Molecular Pathology, Institute of Health Bioscience, The University of Tokushima Graduate School, 3 Kuramotocho, Tokushima 770-8504, Japan, Phone & fax: 81-88-633-7327; E-mail: hayashi@dent.tokushima-u.ac.jp

not known whether disease is initiated by a restrained inflammatory reaction to an organ-specific autoantigen. In most cases, antigenic challenge results in the establishment of immunological memory, a state in which the immune system is maintained to respond effectively upon recurrent antigenic exposure. It is now evident that the interaction of Fas with FasL regulates a large number of pathophysiological processes of apoptosis including autoimmune diseases[7,8]. Recent studies have now confirmed the observation that apoptotic cells in various cell types are implicated as the source of autoantigen when stimulated with different proapoptotic stimuli[9-11]. Apoptosis results in surface accessibility of all SSA/Ro-SSB/La antigens for recognition by circulating maternal antibodies. Although cleavage of certain autoantigens during apoptosis may reveal immunocryptic epitopes that could potentially induce autoimmune responses in systemic autoimmune diseases[12,13], accumulated evidence suggests an important role of apoptosis in the disease pathogenesis of Sjögren's syndrome[14].

**Apoptotic cells in target organs**

Recent studies have suggested that the Fas-FasL system plays a major role in the induction of apoptosis in target organs with autoimmune diseases such as autoimmune gastritis, Hashimoto's thyroiditis, and rheumatoid arthritis (RA)[15-17]. 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[18]. In contrast, expression of Fas by pancreatic $\beta$ cells has been shown to have a major influence on the susceptibility of tissue destruction in nonobese diabetic (NOD) mice to diabetes[19,20]. Since it was reported that Fas expression was observed in the salivary gland cells with human Sjögren's syndrome[21], it is likely that Fas-mediated apoptosis contributes to tissue destruction in the salivary glands with Sjögren's syndrome. A cleavage product of 120 kDa $\alpha$-fodrin was identified as an important autoantigen in human Sjögren's syndrome besides the NFS/*sld* murine model for Sjögren's syndrome[22]. Alpha-fodrin is a ubiquitous, calmodulin-binding protein found to be cleaved by calcium-activated protease (calpain) in apoptotic T cells, and by calpain and/or caspases 3 in anti-Fas-stimulated Jurkat cells and/or neuronal apoptosis[23-26]. It was demonstrated that the fodrin $\alpha$ subunit is cleaved in association with apoptosis, and the 120 kDa fragment is a breakdown product of the mature form of 240 kDa fodrin $\alpha$ subunit[27]. Previous studies have revealed evidence that caspase 3 is required for $\alpha$-fodrin cleavage during apoptosis[28]. In Jurkat cells, caspase 3-like proteases have been reported to cleave $\alpha$-fodrin and poly (ADP-ribose) polymerase (PARP)[29]. In neuroblastoma cells, treatment with staurosporin induced cleavage of $\alpha$-fodrin at both caspase 3 and calpain cleavage sites[30]. *In vitro* studies demonstrated that apoptotic mouse salivary gland (MSG) cells result in a specific $\alpha$-fodrin cleavage into 120 kDa, and preincubation with caspase-inhibitor peptides blocked $\alpha$-fodrin cleavage[31]. A significant increase of TUNEL$^+$-apoptotic epithelial duct cells in the salivary glands was detected in NFS/*sld* Sjögren's syndrome mouse model (Fig. 1). Tissue-infiltrating CD4$^+$ T cells isolated from the salivary gland tissues bear a large proportion of FasL (Fig. 2A & 2B), and MSG cells constitutively express Fas with high proportion. Anti-Fas mAb-stimulated apoptosis in MSG cells was confirmed by flow cytometry of the DNA content of nuclei with PI and Annexin V. Western blot analysis demonstrated that the 240 kDa $\alpha$-fodrin in apoptotic MSG cells was cleaved to smaller fragments into 120 kDa in a time-dependent manner, and the cleavage was entirely blocked by preincubation with caspase inhibitors (z-VAD-fmk, DEVD-CHO) (Fig. 2C). Importantly, the tissue-infiltrating CD4$^+$ T cells, but not CD8$^+$ T cells, are responsible for tissue destruction as judged by *in vitro* $^{51}$Cr release cytotoxic assay against MSG cells *in vitro*. Although it has been reported that Fas-induced apoptosis seems to be the major killing pathway of the CD4$^+$ cytotoxic T cells[32], one mechanism by which activated CD4$^+$ T cells induce cytotoxicity towards salivary gland cells in Sjögren's syndrome is Fas-based. *In vivo* treatment with caspase-inhibitors, z-VAD-fmk and DEVD-CHO, into the murine model results in dramatically inhibited development of autoimmune lesions, and in restoration of sicca syndrome[31]. There is increasing evidence that the cascade of caspases is a critical component of the cell death pathway[33-35], and a few proteins have been found to be cleaved during apoptosis. These include poly (ADP-ribose) polymerase (PARP), a small U1 nuclear ribonucleoprotein (RNP), and $\alpha$-fodrin, which were

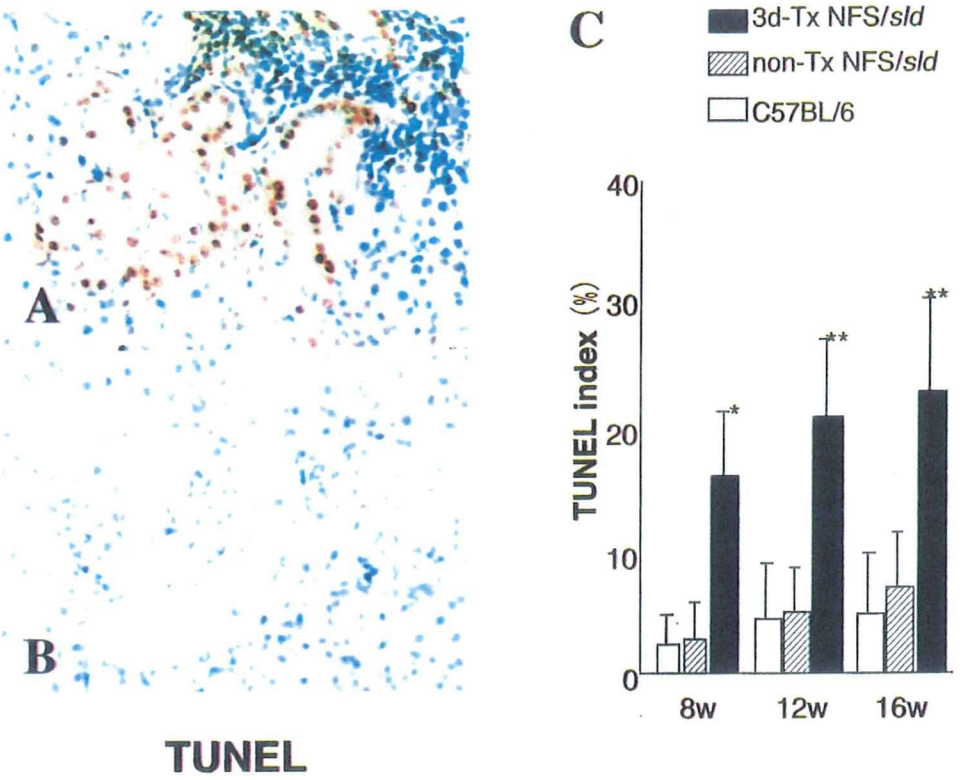



**Fig. 1** Tissue-specific apoptosis in the salivary glands in Sjögren's syndrome mouse model[31]. (A) In situ TUNEL detection was frequently positive for epithelial duct cells in 3d-Tx NFS/*sld* mice (12-wk-old) (A), but not in non-Tx NFS/*sld* mice (12-wk-old) (B). (C) A significant increase of apoptotic duct cells was observed in the salivary gland tissues from 3d-Tx NFS/*sld* mice at all ages. The percentage of duct cells staining positively with TUNEL was enumerated using a 10 × 20 grid net micrometer disc, covering an objective of area 0.16 $mm^2$. Data were analyzed in 10 fields per section, and were expressed as mean percentage ± SD in 5 mice examined per group (asterisks*, $P<0.01$ & asterisks**, $P<0.001$, Student's *t*-test). Five mice in each group were analyzed at 8, 12 and 16 weeks old.

subsequently identified as substrates for caspases[36-38]. The development of autoimmune exocrinopathy in Sjögren's syndrome appears to be dependent on autoantigen cleavage through the caspase cascade, and caspase-inhibitors might provide a new therapeutic option directed at reducing tissue damage.

### Activation-induced cell death (AICD) in Sjögren's syndrome

Activation-induced cell death (AICD) is a well-known mechanism of peripheral T cell tolerance that depends upon an interaction between Fas and Fas ligand (FasL)[39,40]. AICD plays a central role, especially in killing autoreactive T cells and in preventing autoimmune responses[41-43]. It has been reported that activation of T cell clones, or T cell lines, induces FasL expression, and AICD in T cells *in vivo* has been proposed to limit the expansion of an immune response by eliminating effector cells that are no longer needed[44]. Although it is considered that a defect in AICD of effector T cells may result in the development of autoimmune disease[45], the *in vivo* role of organ-specific autoantigen for AICD is entirely unknown. Since the administration of a soluble form of anti-FasL antibody (FLIM58) results in severe destructive autoimmune exocrinopathy in the murine model of Sjögren's syndrome[46], it is possible that an organ-specific autoantigen plays an important role in the down-regulation of AICD. A high titer of serum autoantibodies against 120 kDa $\alpha$-fodrin autoantigen was detected in FLIM58-treated mice, and splenic T cell culture supernatants contained high levels of IFN-$\gamma$. FasL-mediated AICD is down-regulated by autoantigen stimulation in spleen cells from the murine model

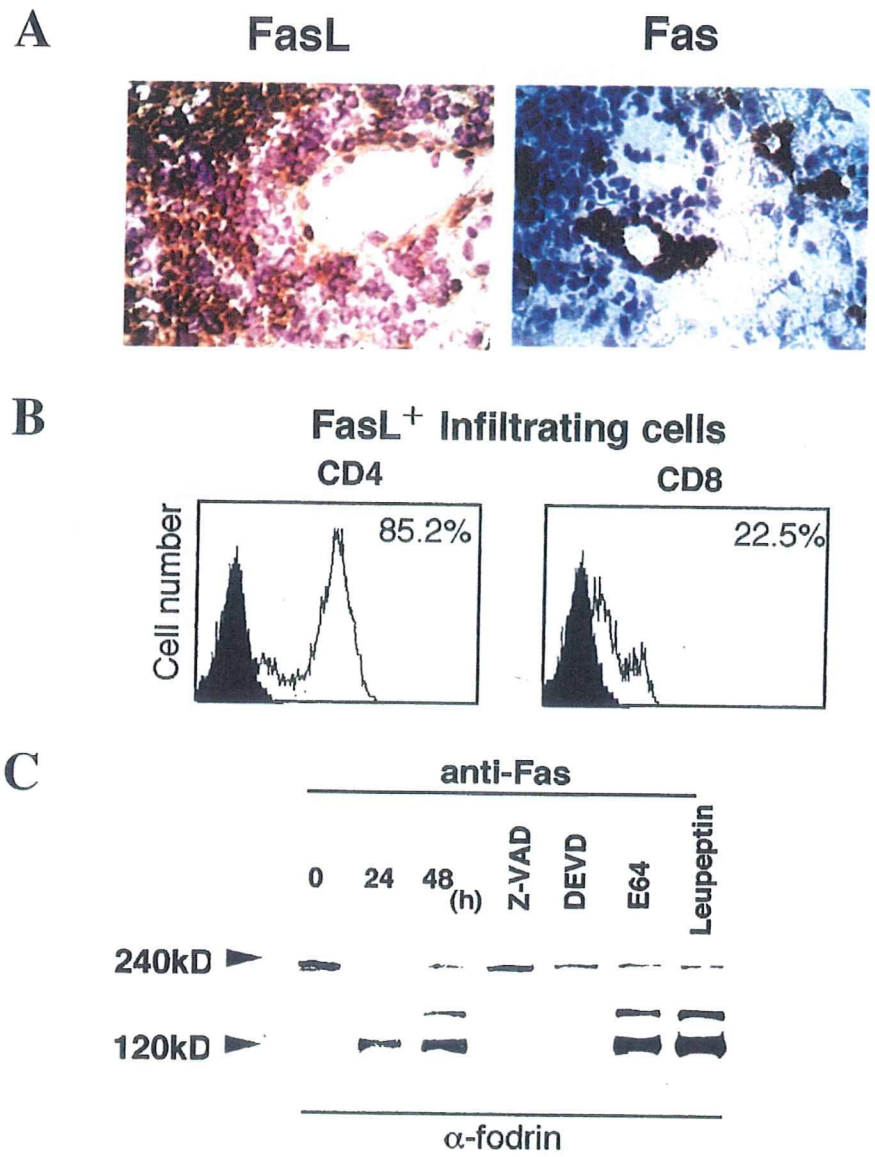



**Fig. 2** FasL and Fas expression on tissue-infiltrating cells in the salivary gland tissues from 3d-Tx NFS/*sld* mice[31]. (A) Immunohistochemical detection of infiltrating lymphocytes strongly positive for FasL in 3d-Tx NFS/*sld* mice. Epithelial duct cells were stained positively with Fas in 3d-Tx NFS/*sld* mice. (B) Flow cytometric analysis of FasL expression on tissue-infiltrating lymphocytes isolated from salivary glands of 3d-Tx NFS/*sld* mice gated on CD4 and CD8. FasL expression on tissue-infiltrating $CD4^+$ T cells was prominent compared with that on $CD8^+$ T cells. (C) Western blot analysis demonstrated that the 240 kDa α-fodrin in apoptotic MSG cells was cleaved to smaller fragments into 120 kDa in a time-dependent manner, and the cleavage product was entirely blocked by preincubation with caspase inhibitors (z-VAD-fmk, DEVD-CHO) measured at 24 h. Protease inhibitor cocktails, cysteine protease inhibitor (E64), and serine protease inhibitor (Leupeptin) had no significant effect on α-fodrin cleavage. The MSG cell apoptosis induced by anti-Fas mAb (*Jo2*) stimulation was determined by flow cytometry of the DNA content of nuclei with PI and Annexin V.