

been reported in SS,²⁸⁻³⁰ the pathogenic roles of the autoantigens in initiation and progression of SS are still unclear.

The specificity of cytotoxic T-lymphocyte function has been an important issue of organ-specific autoimmune response, but little is known about the events triggering T-cell invasion of the target organs in prelude to organ-specific autoimmune diseases. In insulin-dependent diabetes mellitus, the role of environmental factors,^{31,32} the nature of the initiating T cell,^{33,34} and the identity of the inciting antigen(s)³⁵ have all been vigorously debated. When we analyzed the mechanisms of α -fodrin cleavage in the SS salivary glands, infiltrating mononuclear cells bear a large proportion of CD4⁺ and Fas ligand (FasL), and the salivary gland duct cells constitutively possess Fas. In particular, cleavage products of 120-kd α -fodrin were frequently detected in the salivary gland duct cells with SS, but not in control salivary glands. Thus, we provided evidence suggesting that Fas-mediated apoptosis may be involved, in part, in *in vivo* α -fodrin cleavage in SS salivary glands. Moreover, it has been suggested that α -fodrin cleavage triggered by estrogen deficiency plays an important role in the development of autoimmune exocrinopathy in SS. Experimental studies of ours demonstrated a significant apoptosis associated with α -fodrin cleavage in the salivary gland cells of estrogen-deficient healthy C56BL/6(B6) mice, and inflammatory lesions developed exclusively in the salivary and lacrimal gland after the adoptive transfer with α -fodrin-reactive T cells in both ovariectomized (Ovx)-B6 and Ovx-SCID mice.³⁶ Reduction of the intact form of α -fodrin in the affected glands suggests that elicitation of autoreactivity against α -fodrin could be the primary pathogenetic process that leads to tissue destruction. However, based on the fact that α -fodrin is a ubiquitous protein, and that the tissue destruction is confined to exocrine organs, it might be more reasonable to speculate that other undetermined tissue-specific target antigens in exocrine glands could be the primary target of the disease process mediated by pathogenic T cells. Nevertheless, production of autoantibodies and proliferative T-cell responses against cleavage product of α -fodrin, which does not take place under physiological conditions, might be an important clue that could shed light on the novel mechanisms by which tissue-specific apoptosis contributes to the disease development.

It has been reported that calpain is overactivated in autoimmune conditions, and subsequent tissue destruction.^{37,38} Moreover, the cascade of caspases is a critical component of the cell death pathway,³⁹⁻⁴¹ 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.^{27,42} Anti-Fas-induced cleavage of α -fodrin in Jurkat cells produces a predominant 120-kd fragment, and the 120-kd fragment is consistent with a previously reported caspase 3-mediated DETD cleavage site within the protein.^{20,21} Although the relevance

of cleavage of structural proteins, including gelsolin, actins, lamins, and fodrins, is easily conceivable,⁴³ the functional importance is not yet clear. Our data provide evidence that α -fodrin in human HSY cells is cleaved into 120-kd fragment by apoptotic proteases including calpain and caspases. When we investigated whether cysteine proteases are involved in α -fodrin cleavage, anti-Fas-treated HSY cells were positive for mAb to μ -calpain, and caspase 3 in association with apoptosis. However, we demonstrated here that calpastatin, an endogenous inhibitor of calpain, was shown to be constitutively expressed, speculating that μ -calpain activity could be considerably affected during apoptosis in HSY cells. A combination of calpain inhibitor peptide and caspase inhibitors (Z-VAD-fmk) entirely blocked the formation of 120-kd α -fodrin. When both full-length caspase 3 and μ -calpain cDNAs were transiently overexpressed in HSY cells, a cleavage product of 120-kd α -fodrin was abundantly identified than the level of expression of caspase 3 or μ -calpain in cells transfected with each construct. These data suggest that both μ -calpain and caspase 3 are required for specific α -fodrin proteolysis into the 120-kd fragment in human salivary gland cells with SS.

In this study, we detected proliferative T-cell responses to AFN protein (JS-1) of SS patients with short duration (within 5 years) from the onset of the disease were significantly higher than those with long duration (more than 5 years). Proliferative responses to autoantigen with younger SS patients (40 to 50 years of age) were significantly higher than those with older SS patients (60 to 70 years of age). Moreover, significant proliferative responses to AFN protein were observed in SS patients with higher pathological score (grade 4) than those with lower score (grade 2 or grade 3). These data are suggestive of clinicopathological usefulness of AFN immunoreactivity with SS patients for disease severity in addition to diagnostic significance. Because we have detected proliferative response to AFN peptides using PBMCs from SS patients, it is feasible for the future possibility that a peptide analogue of AFN could be used as a therapeutic agent. On the other hand, Fas/FasL interaction down-regulates the immune response by inducing apoptosis because activated lymphocytes express both Fas and FasL.⁴⁴ CD4⁺ T cells from PBMCs with SS patients induce Th1 cytokine (IL-2, IFN- γ) when pulsed with each peptide, suggesting that the autoantigen peptide may play an important role in Th1/Th2 balance *in vivo*. Moreover, AFN peptide-pulsed CD4⁺ T cells down-regulate Fas-mediated apoptosis. Although antigen-induced T-cell death is known to be regulated by CD4 expression,⁴⁵ molecular mechanisms responsible for T-cell death should be further elucidated. Our previous findings support the notion that Fas-mediated T-cell death is down-regulated by autoantigen stimulation in the murine SS model.⁴⁶ Here we demonstrated that AFN peptide stimulation results in a significant decrease in anti-Fas-induced CD4⁺ T-cell apoptosis. However, it remains unclear whether T cells specific for endogenous

epitopes play a significant pathological role in tissue damage during the clinical episodes.

Taken together, our results suggest that 120-kd α -fodrin, the apoptosis-associated breakdown product, may have an important role in the development of SS, and that the autoantigen is a novel participant in the regulation of Th1/Th2 balance and peripheral tolerance.

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

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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 keratoconjunctivitis 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,

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caspace, 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 neopeptide. 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 colleagues²⁴ 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 μ g/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 μ g/ml) and ovalbumin (4 μ g/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 γ 1 (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 *NheI* and *SpeI* 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% CO₂ 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 μ g/ml of cycloheximide for 3 to 15 hours at 37°C/5% CO₂. 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- μ m 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 μ g/ml in PBS), or mouse

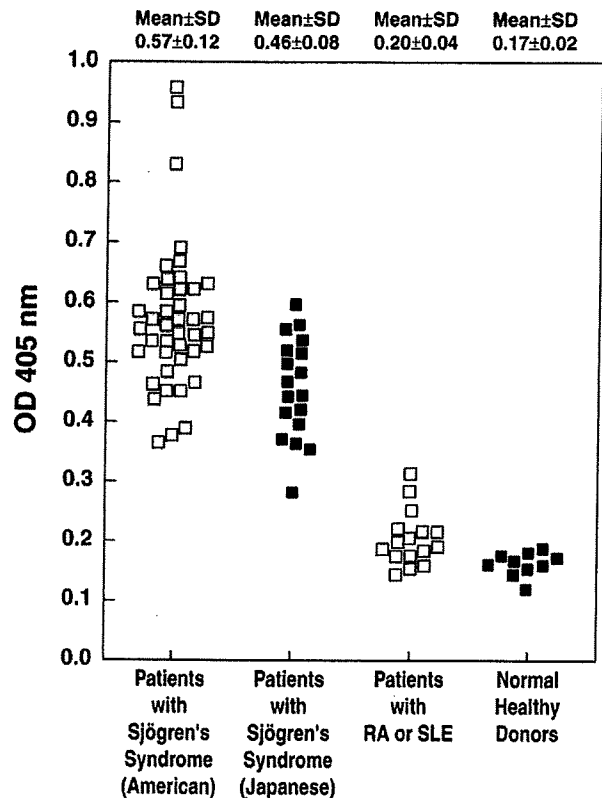


Figure 1. Sera from SS patients contain anti- α -fodrin Abs, as measured by ELISA. Sera, diluted 1:400 in PBS, from 42 American SS patients, 17 Japanese SS patients, 16 RA and SLE patients, and 10 healthy individuals were tested for binding to mouse brain α -fodrin by ELISA. Samples with A₄₀₅ values more than twice the mean of the control normals (>0.33) were considered positive.

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 iodide (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.

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 OD₄₀₅ 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 (OD₄₀₅ nm, 0.57 ± 0.12) and Japanese SS patients (OD₄₀₅ 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 (OD₄₀₅ 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.

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, IgG₁ 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 ($\approx 6 \times 10^6$ members) and SS30

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.

($\approx 8 \times 10^6$ 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

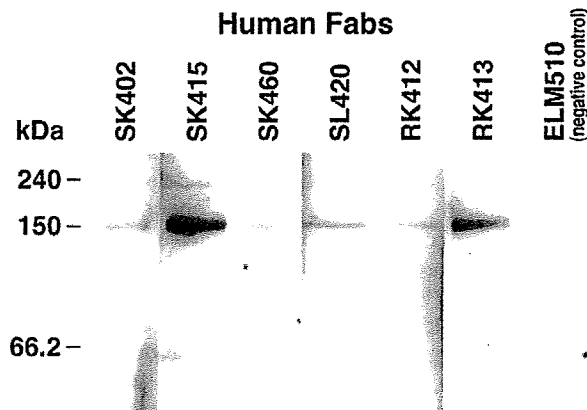


Figure 2. Binding of human monoclonal IgG Fabs to purified mouse α -fodrin brain extract by Western blot analysis. All of the Fabs specifically bound to the 150-kd fragment of cleaved α -fodrin and did not react with 240-kd intact α -fodrin. The human monoclonal anti-Ebola virus Fab ELZ510 was used as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

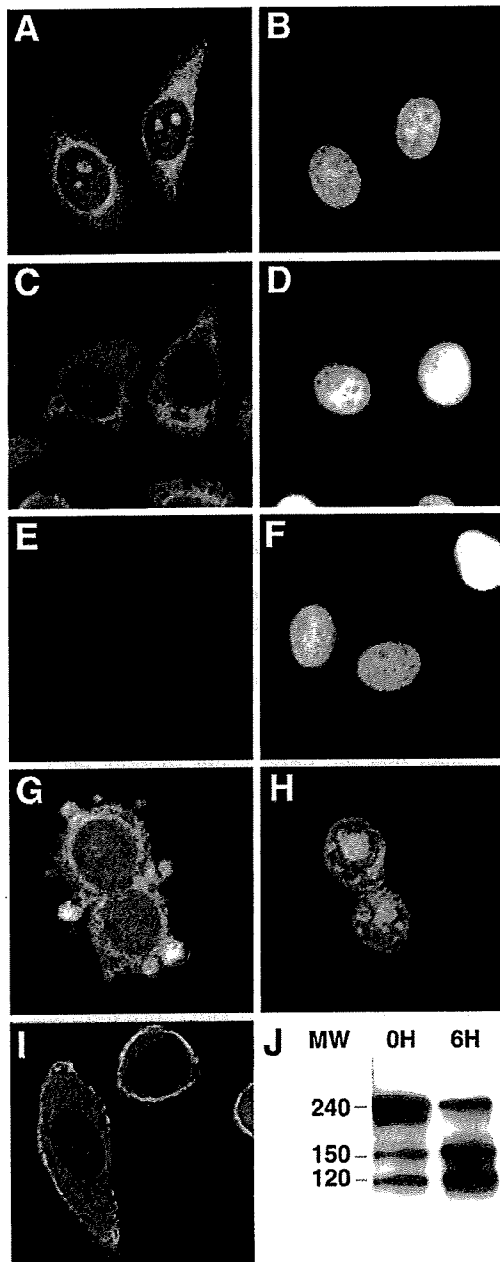


Figure 3. Subcellular distribution of intact and cleaved α -fodrin in human salivary cells. Ethanol-fixed human salivary HSG cells were stained with the human anti-150-kd α -fodrin Fabs SK415 (A) and SK460 (C), a human anti-HIV-1 gp120 Fab, b12 (E, negative control), and the mouse anti- α -fodrin mAb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

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

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

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

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

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

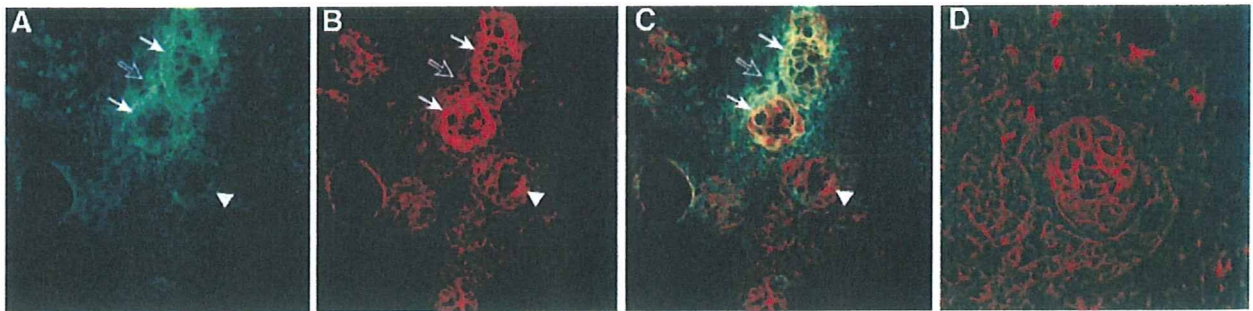


Figure 4. Distribution of cleaved and intact α -fodrin in salivary gland tissue of a SS patient with active disease. Tissue sections from salivary gland biopsies of patients with SS were stained with human anti-150-kd α -fodrin Fab SK415 (A, green) and mouse anti- α -fodrin mAb AA6 (B, red) and analyzed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

Discussion

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

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

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

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

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

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

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

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Estrogen Deficiency Accelerates Murine Autoimmune Arthritis Associated with Receptor Activator of Nuclear Factor- κ B Ligand-Mediated Osteoclastogenesis

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The aims of this study were to evaluate the *in vivo* effects of estrogen deficiency in MRL/*lpr* mice as a model for rheumatoid arthritis and to analyze the possible relationship between immune dysregulation and receptor activator of nuclear factor- κ B ligand (RANKL)-mediated osteoclastogenesis. Experimental studies were performed in ovariectomized (Ovx)-MRL/*lpr*, Ovx-MRL+/+, sham-operated-MRL/*lpr*, and sham-operated-MRL+/+ mice. Severe autoimmune arthritis developed in younger Ovx-MRL/*lpr* mice until 24 wk of age, whereas these lesions were entirely recovered by pharmacological levels of estrogen administration. A significant elevation in serum rheumatoid factor, anti-double-stranded

DNA, and anti-type II collagen was found in Ovx-MRL/*lpr* mice and recovered in mice that underwent estrogen administration. A high proportion of CD4⁺ T cells bearing RANKL was found, and an enhanced expression of RANKL mRNA and an impaired osteoprotegerin mRNA was detected in the synovium. An increase in both osteoclast formation and bone resorption pits was found. These results indicate that estrogen deficiency may play a crucial role in acceleration of autoimmune arthritis associated with RANKL-mediated osteoclastogenesis in a murine model for rheumatoid arthritis. (*Endocrinology* 145: 2384–2391, 2004)

IT IS WELL KNOWN that sex steroids have significant impact on the development of autoimmune diseases in both humans and rodents. In particular, estrogen has been suggested to be responsible for the strong female preponderance of the human rheumatoid arthritis (RA), systemic lupus erythematosus, scleroderma, and Sjögren's syndrome (1–3), but the role of estrogens in the female has not been fully characterized. RA is a chronic inflammatory disease characterized by invasive synovial hyperplasia leading to progressive joint destruction. Rheumatoid synovial cells are not only morphologically characterized by their transformed appearance (4) but also are phenotypically transformed to proliferate abnormally (5, 6). These cells invade bone and cartilage by producing an elevated amount of proinflammatory cytokines (7) and matrix metalloproteinases (MMPs) (8) and by inducing osteoclast (OC) formation and activation (9, 10).

OCs, the multinucleated cells exclusively responsible for bone resorption, have been observed to resorb bone actively at the site of invasion of the proliferated synovial membrane into the adjacent bone (11). The cell types responsible for bone resorption in RA have been characterized as authentic

OCs (12), and it was reported that rheumatoid synovial fibroblasts are involved in bone destruction by inducing osteoclastogenesis (13). However, the exact mechanisms involved in the formation and activation of OCs in RA are still unclear.

Receptor activator of nuclear factor- κ B ligand (RANKL) (14) is a regulator of the immune system and of bone development (15). RANKL is expressed on activated T cells (16), and a major target for RANKL in the immune system appears to be mature dendritic cells (DCs) that express a high level of RANKL receptor (RANK) (17). *In vitro*, RANKL promotes the survival of mature DCs, most likely by up-regulating the expression of Bcl-XL (18), and induces the production of proinflammatory cytokines, such as IL-1 and IL-6, and cytokines that stimulate and induce differentiation of T cells, such as IL-12 and IL-15 (19). Therefore, RANKL is likely to act as a positive-feedback regulator during productive T cell-DC interactions (20).

The MRL/*lpr* mouse strain was chosen to test the estrogenic action because it has a genetic predisposition to arthritis with characteristics similar to those of human RA including cell infiltration, pannus formation, bone and cartilage breakdown, and the presence of serum rheumatoid factor (RF) (21–23). The aim of this study was to analyze the *in vivo* effects of estrogen deficiency on the development of autoimmune arthritis in MRL/*lpr* mice and to evaluate the possible relationship with RANKL-mediated osteoclastogenesis.

Materials and Methods

Mice and treatment

MRL/Mp-*lpr/lpr* (MRL/*lpr*, age 4–24 wk; n = 108) and MRL+/+ mice (age 4–24 wk; n = 58) were purchased from Charles River Japan

Abbreviations: Ab, Antibody; CII, type 2 collagen; DC, dendritic cell; dsDNA, double-stranded DNA; ER, estrogen receptor; IFN, interferon; IRF, IFN regulatory factor; LN, lymph node; mAb, monoclonal antibody; MMP, matrix metalloproteinase; OC, osteoclast; OPG, osteoprotegerin; Ovx, ovariectomized; RA, rheumatoid arthritis; RANK, RANKL receptor; RANKL, receptor activator of nuclear factor- κ B ligand; RF, rheumatoid factor; sham, sham-operated; TRAP, tartrate-resistant acid phosphatase.

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Inc. (Atsugi, Japan), and were fed under specific pathogen-free conditions. Female MRL/lpr mice and MRL+/+ mice (4 wk of age) were ovariectomized (Ovx) and compared with sham-operated (sham)-MRL/lpr and MRL+/+ mice. Six to 10 mice in each group were analyzed at 8, 12, 16, 20, and 24 wk of age. Ovx-MRL/lpr mice were administered im with 60 mg/kg-wk estrogen (Ovahormine depo; Teikoku Zouki Inc., Tokyo, Japan) in sesame oil or sc with 25 mg/kg-d testosterone (Wako Pure Chemical, Osaka, Japan) from 4–20 wk of age. Care of the mice was in accordance with institutional guidelines.

Histology and immunohistology

All organs were removed from the mice and fixed with 10% phosphate-buffered formalin, and ankles were further decalcified in 10% EDTA. Sections (4 μ m in thickness) were stained with hematoxylin and eosin. Histological grading of inflammatory arthritis was done according to the methods by Edwards et al. (24) as follows: one point score indicates hyperplasia/hypertrophy of synovial cells; fibrosis/fibroplasia; proliferation of cartilage and bone; destruction of cartilage and bone; and/or mononuclear cell infiltrate. Immunohistological analysis was performed on freshly frozen sections (4 μ m in thickness) by the biotin-avidin immunoperoxidase method using ABC reagent (Vector Laboratories Inc., Burlingame, CA). The monoclonal antibodies (mAb) used were biotinylated rat mAbs to CD4, CD8 (BD Biosciences, San Jose, CA), and mouse RANKL (IMGENEX, San Diego, CA).

Flow cytometric analysis

Spleen and inguinal lymph node (LN) cell suspensions were stained with antibodies (Ab) conjugated to phycoerythrin (anti-CD4, Cedarlane Laboratories Ltd., Ontario, Canada; B220, PharMingen, San Diego, CA), fluorescein isothiocyanate (anti-CD8, Cedarlane Laboratories; Thy1.2, PharMingen), and antimouse RANKL (IMGENEX) and analyzed with EPICS (Coulter, Miami, FL).

Measurement of anti-double-stranded DNA (dsDNA) Ab, RF, and type 2 collagen (CII) Ab levels

Anti-dsDNA Abs, RF, and anti-CII Ab were detected by ELISA as described previously (25–27). Briefly, flat-bottom plates (Nalge Nunc International, Roskilde, Denmark) were coated with 1.5 μ g/ml of native calf thymus DNA (Life Technologies, Inc., Rockville, MD) in buffer containing 0.1 M sodium bicarbonate and 0.05 M citric acid at 4 C overnight. Serum samples were serially diluted (starting at 1/200) and added to the plates for a 1-h incubation at 37 C. After washing, peroxidase-conjugated goat antimouse IgG, or IgM (Southern Biotechnology Associates, Birmingham, AL), was added and incubated for 1 h at 37 C. Ab binding was visualized using orthophenylenediamine (Sigma, St. Louis, MO). For the measurement of IgG and IgM RF, human IgG and IgM (Chemicon International, Temecula, CA) were coated onto plates at 10 μ g/ml in carbonate buffer, and the same procedures were followed as described above. For the measurement of serum Abs to CII, native bovine CII was dissolved in 0.1 M acetic acid at 1 mg/ml and diluted with 0.1 M sodium bicarbonate at 10 μ g/ml (pH 9.6). The microtiter plate was coated with 100 μ l of CII antigen solution. After washing three times, 100 μ l per well of serum samples that had been serially diluted in PBS/Tween 20/1% BSA and control serum were added and incubated for 1 h at 37 C. After washing, peroxidase-conjugated goat antimouse IgG (at 1.4 μ g/ml, 100 μ l per well) (Organon Teknika, Durham, NC) was added and incubated for 1 h at 37 C. A total of 100 μ l o-phenylenediamine (0.5 mg/ml) dissolved in 0.1 M citrate buffer (pH 5.0) containing 0.012% H₂O₂ was added, and the reaction was stopped using 8 N H₂SO₄ (20 μ l per well).

Measurement of cytokine production

Cytokine production was tested by two-step sandwich ELISA using a mouse IL-2, IL-4, and interferon (IFN)- γ kit (Genzyme, Cambridge, MA). In brief, culture supernatants from LN cells activated with immobilized anti-CD3 mAb (Cedarlane Laboratories) for 3 d were added to microtiter plates precoated with anti-IL-2, IL-4, and IFN- γ capture Ab and incubated overnight at 4 C. After addition of biotinylated detecting Ab and incubation at room temperature for 45 min, avidin-peroxidase

was added and incubated at room temperature for 30 min. Finally, 2,2'-azino-di-3-ethylbenzthiazoline sulfonate substrate containing H₂O₂ was added, and the colorimetric reaction was read at an absorbance of 405 nm using an automatic microplate reader (Bio-Rad Laboratories Inc., Hercules, CA). The concentrations of IL-2 (picograms per milliliter), IL-4 (picograms per milliliter), and IFN- γ (picograms per milliliter) were calculated according to the standard curves produced by various concentrations of recombinant cytokines.

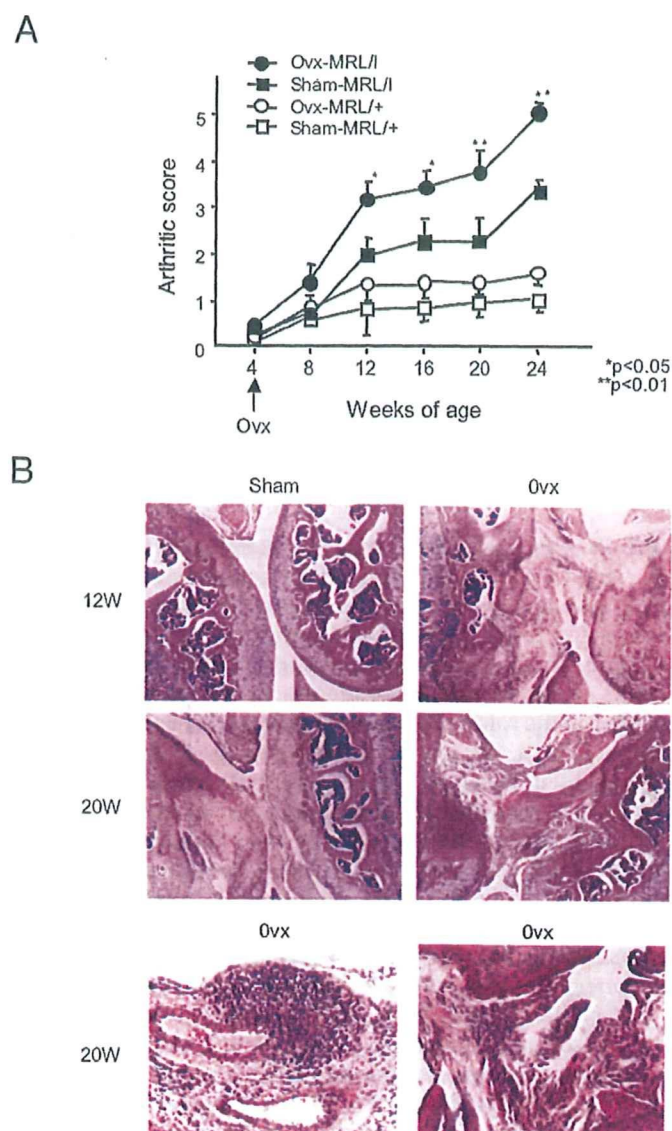


FIG. 1. Effects of the Ovx on joint histopathology. Histological score of autoimmune arthropathy developed in younger Ovx-MRL/lpr mice compared with those in sham-MRL/lpr and Ovx-MRL+/+ mice until 24 wk of age (A). Histological evaluation of the knee joints was performed according to the methods by Edwards et al. (24) (*, $P < 0.05$; and **, $P < 0.01$, Student's t test). Representative photomicrographs taken from Ovx- and sham-MRL/lpr mice at 12 (12W) and 20 wk (20W) of age (B). The histopathological effects observed in Ovx-MRL/lpr mice at age 20 wk included mononuclear cell infiltration into the subsynovial tissue (lower left), and synovial hyperplasia (lower right) (hematoxylin and eosin). In contrast, mononuclear cell infiltration and bone and cartilage pathology was absent in sham-MRL/lpr mice until 20 wk of age (middle left).

ELISA for murine osteoprotegerin (OPG)

An anti-murine OPG mAb (TECHNE Corp., Minneapolis, MN) was diluted with 0.1 M sodium bicarbonate (pH 9.6) solution to a concentration of 10 $\mu\text{g}/\text{ml}$, and the 100- μl aliquot was added to each well of 96-well plates. After incubation at 4 C overnight, the capture solution was removed by flicking the plates, and the wells were blocked with the blocking solution (300 μl per well) for 2 h at room temperature. Recombinant OPG (100 μl) (R&D System Inc., Minneapolis, MN) standard and a series of test samples were added to the wells, and the plates were incubated for 2 h at room temperature. The wells were then washed with the washing buffer, and 100 μl of peroxidase-labeled anti-OPG mAb was added to each well. After incubation for an additional 2 h, 100 μl of tetramethylbenzidine substrate reagent was added to each well. Tetramethylbenzidine stop buffer (100 μl) was added to each well, and absorbance at 450 nm of the wells was measured with a microplate reader (Bio-Rad Laboratories Inc.).

RT-PCR

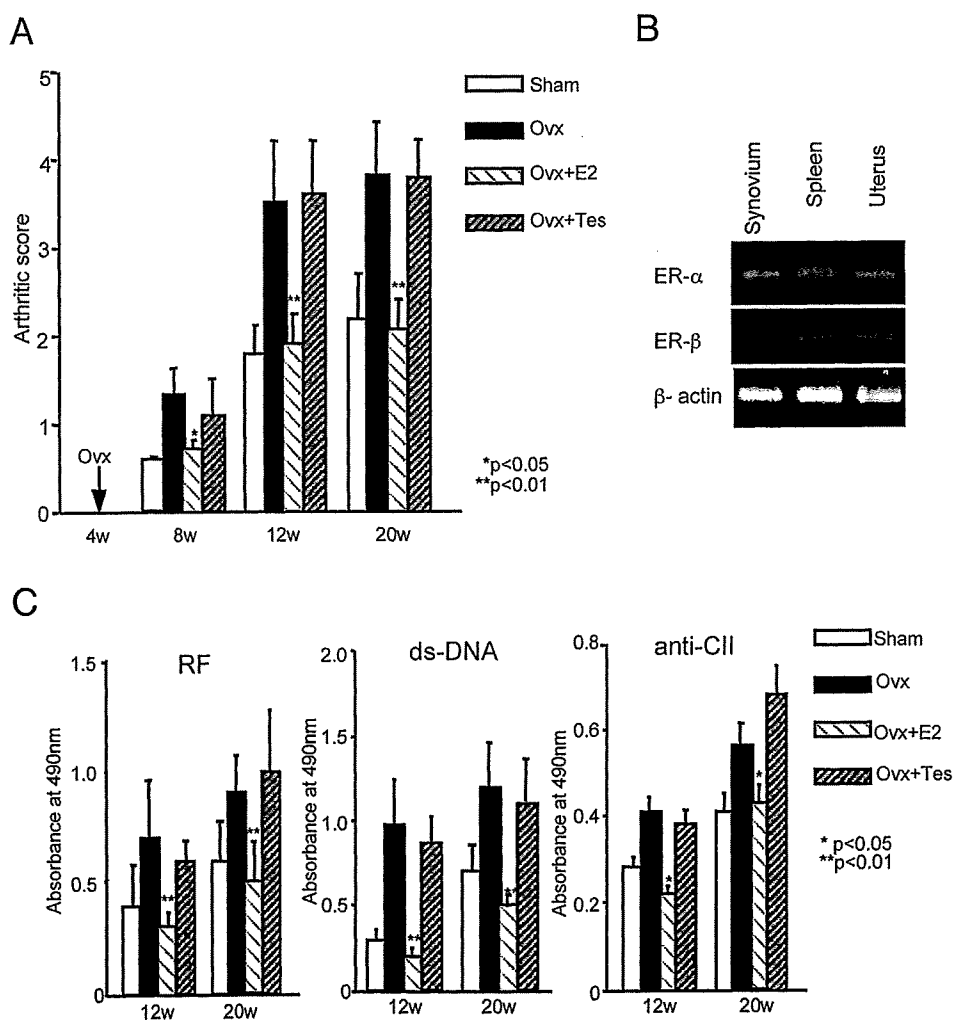
The total RNA from LNs and synovial tissues was extracted as reported previously (28). The RNA was reverse-transcribed into cDNA. The cDNA reaction mixture was diluted with 90 μl of PCR buffer and mixed with 500 nm of the 5' and 3' primers, 0.1 mM deoxynucleotide triphosphate mix, 2 mM MgCl_2 , and 2 U thermostable *Taq* polymerase (PerkinElmer Cetus, Norwalk, CT). The cDNA was subjected to enzymatic amplification in a DNA thermal cycler (PerkinElmer Cetus) by using specific primers. PCR was carried out at 55 C annealing temperature for 30–35 cycles. The specific primers used were as follows: IL-1 β , TGA TGA GAA TGA CCT GTT CT and CTT CTT CAA AGA TGA AGG

AAA; TNF- α , ATG AGC ACA GAA AGC ATG ATC and AGA TGA TCT GAG TGT GAG GG; IL-6, CTC TGC AAG AGA GAC TTC CAT and ATA GGC AAA TTT CCT GAT TAT A; IFN- γ , CCT CAG ACT CTT TGA ACT CT and CAG CGA CTC CTT TTC CGC TT; IFN regulatory factor (IRF)-1, TCT GAG TGG CAT ATG CAG ATG GAC and GGT CAG AGA CCC AAA CTA TGG TCG; MMP-1, ATG GTG GGG ATG CCC ATT TT and CAG CAT CTA CTT TGT TGC C; MMP-2, GAG TTG GCA GTG CAA TAC CT and GCC ATC CTT CTC AAA GTT GT; MMP-3, GAA ATG CAG AAG TTC CTC GG and GAG TTC CAT AGA GGG ACT GA; MMP-9, CCA TGA GTC CCT GGC AG and AGT ATG TGA TGT TAT GAT G; TIMP (tissue inhibitor of metalloproteinase)-1, CTG GCA TCC TCT TGT TGC TA and AGG GAT CTC CAA GTG CAC AA; RANKL, GGG AAT TAC AAA GTG CAC CAG and GCC ATC CTT CTC AAA GTT GT; RANK, GTC TTC TGG AAC CAT CTT CTC C and CAC AGA CAA ATG CAA ACC TTG; OPG, TCA AGT GCT TGA GGG CAT AC and TGG AGA TCG AAT TCT GCT TG; estrogen receptor (ER)- α , AAT TCT GAC AAT CGA CGC CAG and GTG CTT CAA CAT TCT CCC TCC TC; ER- β , TTC CCA GCA CCA CCG GTA ACC T and TCC CTC TTG GCG CTT GGA CTA; β -actin, GTG GGC CGC TCT AGG CAC CA and CGG TTG GCC TTA GGG TTC AGG GGG. The amplified DNA reaction mixture was subjected to 1.7% agarose gel electrophoresis, and the amplified product was visualized by UV fluorescence after staining with ethidium bromide.

Tartrate-resistant acid phosphatase (TRAP) staining

Staining for TRAP was performed according to the modified method of Takahashi *et al.* (29). Sections were rinsed once with PBS (pH 7.4), air dried, fixed with 10% formalin in PBS (pH 5.4) for 5 min, and fixed with

FIG. 2. The destructive lesions in the knee joints in Ovx-MRL/lpr mice were inhibited by estrogen administration (10^{-9} M) at ages 12 and 20 wk (*, $P < 0.05$ and **, $P < 0.01$, Student's *t* test) (A). Detection of gene expression in ER- α in the synovium but not in ER- β by RT-PCR analysis (B). E2, 17 β -Estradiol. Serum RF, anti-dsDNA Abs, and anti-CII were significantly increased in Ovx-MRL/lpr mice compared with those in sham-MRL/lpr mice, and these changes were entirely recovered by the treatment with estrogen administration (10^{-9} M) at 12 and 20 wk of age (*, $P < 0.05$; and **, $P < 0.01$, Student's *t* test) (C). Tes, Testosterone.



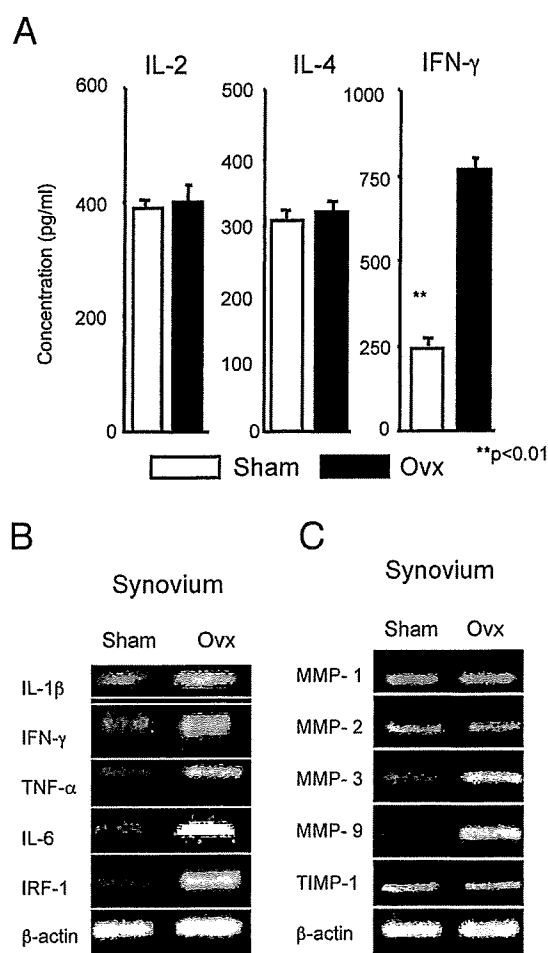


FIG. 3. Effects of the Ovx on expression of cytokines and MMPs. Culture supernatants from anti-CD3 mAb-stimulated LN T cells obtained from Ovx-MRL/*lpr* mice at age 20 wk contained high levels of IFN- γ (**, $P < 0.01$, Student's *t* test), whereas no different levels of IL-2 and IL-4 was observed by ELISA (A). Increased expressions of cytokine genes including IL-1 β , IFN- γ , TNF- α , IL-6, IRF-1, and β -actin were detected in synovium from Ovx-MRL/*lpr* mice at age 20 wk, compared with those from sham-mice by RT-PCR analysis (B). Enhanced gene expressions of MMP-3, and MMP-9 mRNA were found in synovium from Ovx-MRL/*lpr* mice at age 20 wk (C).

methanol-acetone (50:50; pH 5.4) for 30 sec. The coverslips were air-dried and stained for 15 min at room temperature in a 0.1 M sodium acetate buffer (pH 5.0) containing naphthol AS-MX phosphate (Sigma) as a substrate and fast red violet LB salt (Sigma) as a stain for the reaction product in the presence of 50 mM of sodium tartrate.

Assessment of bone resorption

Bone marrow cells (5×10^5) from Ovx- and sham-MRL/*lpr* mice were added to the wells of 96-well plates containing a slice of bovine cortical bone and incubated in a total volume of 200 μ l α -MEM-fetal bovine serum as described previously (30). All cultures were maintained in the presence of dexamethazone (10^{-7} M, FUNAKOSHI Pharmacol., Tokyo, Japan) and $1\alpha,25$ -(OH) $_2$ D $_3$ (10^{-8} M, Chugai Inc., Tokyo, Japan) for 10 d. Bone slices were assessed for bone resorptive activity, brushed with a rubber policeman to remove cells after observation under a microscope, and stained with Mayer's hematoxylin. Bone resorption pits were quantified by densitometric analysis of images of the whole area of bone slices as previously described (30). Additionally, *in vitro* osteoclastogenesis was assayed using bone marrow-derived TRAP-positive cells with macrophage colony stimulating factor (5 ng/ml; PeproTech EC, London,

UK), recombinant murine RANKL (10 ng/ml; PeproTech Inc., Rocky Hill, NJ), recombinant OPG (100 ng/ml; R&D Systems Inc.), and 17β -estradiol (10^{-9} M), at indicated concentration after the estimation of dose responses.

Results

Effects of the Ovx on joint histopathology

Destructive autoimmune arthritis developed in young Ovx-MRL/*lpr* mice, not in young sham-MRL/*lpr* and Ovx-MRL+/+ mice, and these lesions aggravated with age from 12 until 24 wk of age. Histological analysis of the knee joints was performed at 8, 12, 16, 20, and 24 wk of age for all the experiments. Analysis of the histological results for the experiment, shown in Fig. 1A, indicates that the group that was treated with Ovx had significant higher subsynovial inflammation, synovial hyperplasia, pannus formation and cartilage erosion, bone destruction, and overall histopathology. Shown in Fig. 1B are photomicrographs taken from representative Ovx- and sham-MRL/*lpr* mice at 12 and 20 wk of age. The effects observed in Ovx-MRL/*lpr* mice included synovial hyperplasia, pannus formation, bone erosion, and infiltration of mononuclear cells into the subsynovial tissue. In contrast, mononuclear cell infiltration and bone and cartilage pathology was absent in sham-MRL/*lpr* mice until age 20 wk.

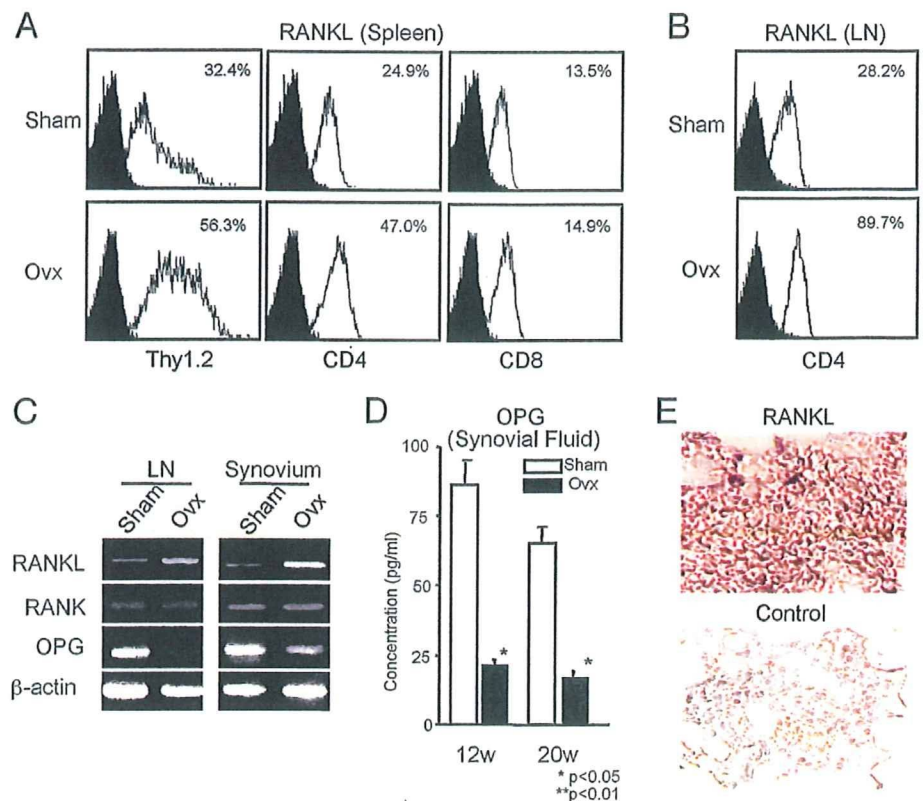
Recovery of autoimmune arthritis by estrogen administration

The destructive lesions in the knee joints in Ovx-MRL/*lpr* mice were inhibited by estrogen administration (10^{-9} M) at 12 and 20 wk of age (Fig. 2A). Testosterone administration in Ovx-MRL/*lpr* mice resulted in severe inflammatory lesions as the same levels. We confirmed gene expression in ER- α but not ER- β in synovial tissues by RT-PCR analysis (Fig. 2B), indicating that estrogenic action to the synovial tissues might be directly affected through estrogen/ER- α binding *in vivo*. We detected increased levels of serum RF, anti-dsDNA, and anti-CII Abs in Ovx-MRL/*lpr* mice compared with those in sham mice, and these levels were entirely recovered in Ovx-MRL/*lpr* mice that underwent estrogen administration (Fig. 2C).

Effects of the Ovx on cytokine and MMP expression

Culture supernatants from anti-CD3 mAb-stimulated LN T cells obtained from Ovx-MRL/*lpr* mice at 20 wk of age contained high levels of IFN- γ , whereas no difference in levels of IL-2 and IL-4 was observed by ELISA (Fig. 3A). We next analyzed the effects of the Ovx on various gene expressions in the synovial tissues. Increased expressions of cytokine genes including IL-1 β , IFN- γ , TNF- α , IL-6, IRF-1, and β -actin mRNA were detected in synovial tissues from Ovx-MRL/*lpr* mice at 20 wk of age, compared with those from sham-MRL/*lpr* mice by RT-PCR analysis (Fig. 3B). In addition, we found elevated gene expressions of MMP-3 and MMP-9 in synovial tissues from Ovx-MRL/*lpr* mice (Fig. 3C). These data suggest that estrogen deficiency induces various gene expressions directly responsible for tissue damage on the development of autoimmune arthritis.

FIG. 4. A significant increase in splenic Thy1.2⁺ and CD4⁺ T cells bearing RANKL from Ovx-MRL/lpr mice at 20 wk of age was observed as compared with those from sham-MRL/lpr mice, whereas no remarkable change in CD8⁺ T cells bearing RANKL was found (A). A large proportion of CD4⁺ T cells in LN bearing RANKL (89.7%) from Ovx-MRL/lpr mice was detected on flow cytometry, as compared with that of sham-MRL/lpr mice (28.2%) (B). A prominently enhanced RANKL mRNA and an impaired OPG mRNA were observed in LN and synovium from Ovx-MRL/lpr mice by RT-PCR analysis (C). A significantly decreased OPG concentration was found in synovial fluid of Ovx-MRL/lpr mice at ages 12 (12w) and 20 wk (20w) (*, $P < 0.05$; **, $P < 0.01$, Student's *t* test) (D). A large proportion of infiltrating cells in synovium was positive for RANKL in Ovx-MRL/lpr mice at 20 wk of age. Isotype-matched controls were all negative (E).



Effects of the Ovx on RANKL, RANK, and OPG expression

We analyzed the spleen and LN cells bearing RANKL by flow cytometry. A significant increase of Thy1.2⁺, and CD4⁺ T cells bearing RANKL in the spleen from Ovx-MRL/lpr mice was observed, compared with those from sham-MRL/lpr (Fig. 4A). We detected a large proportion of CD4⁺ T cells in LN bearing RANKL (89.7%) from Ovx-MRL/lpr mice as compared with those from sham-MRL/lpr mice (28.2%) (Fig. 4B). In addition, an enhanced RANKL mRNA and an impaired OPG mRNA were observed in LN and synovium from Ovx-MRL/lpr mice, compared with those in sham-mice by RT-PCR analysis (Fig. 4C). Indeed, a significant decrease in OPG concentration was found in synovial fluid of Ovx-MRL/lpr mice compared with those of sham-mice at 12 and 20 wk of age (Fig. 4D). A large proportion of infiltrating cells in synovium was positive for RANKL in Ovx-MRL/lpr mice (Fig. 4E).

Effects of the Ovx on OC formation and bone resorption

As seen in Fig. 5A, numerous multinucleated (more than three nuclei), TRAP-positive OC-like cells were detected in the knee joints from both Ovx- and sham-MRL/lpr mice. A significant increase in number of OCs formed in the joints from Ovx-MRL/lpr mice was observed compared with those from sham-MRL/lpr mice (Fig. 5B). We next examined *in vitro* whether the TRAP-positive multinucleated cells (more than three nuclei) from bone marrow cells resorbed bovine bone slices (30). When the bone marrow cells were cultured with dexamethasone and $1\alpha,25\text{-(OH)}_2\text{D}_3$, they differentiated into TRAP-positive multinucleated cells, and numerous resorption pits were formed on their surfaces (Fig. 5C). A

significantly large number of resorption pits were formed using the bone marrow cells from Ovx-MRL/lpr mice compared with those from sham-MRL/lpr mice (Fig. 5D).

Discussion

RA is characterized by progressive joint damage that is mediated by several mechanisms (31). Although the etiology of RA remains unknown, joint damage results from the degradation of connective tissue by MMPs and the stimulation of osteoclastogenesis by activated CD4⁺ T cells (10). Sex hormones influence both humeral and cell-mediated immune response, and estrogen is one of potential factors in this immunological dimorphism (32–34). We have examined the effects of the Ovx on the development of autoimmune arthritis in animals that are susceptible to the development of human RA-like disease. Histology of autoimmune arthritis in Ovx-MRL/lpr mice showed severe destructive changes in the younger age examined. It is generally accepted that a severe inflammatory arthritis and systemic autoimmune disease, including glomerulonephritis and autoantibody production, develop in aged (>6-month-old) MRL/lpr mice (21, 22). We show here that estrogen deficiency caused a severe inflammatory arthritis in younger (<3-month-old) MRL/lpr mice, and these lesions were dramatically prevented by exogenous estrogen treatment. Although the mechanism in detail of estrogen activity still remains unclear, these findings suggest that estrogenic action has an important role during development of autoimmune arthritis in MRL/lpr mice. An estrogen deficiency by Ovx in murine RA model results in a significant increase of serum RF, anti-dsDNA Abs, and anti-CII Ab, and these changes were recovered by estrogen ad-

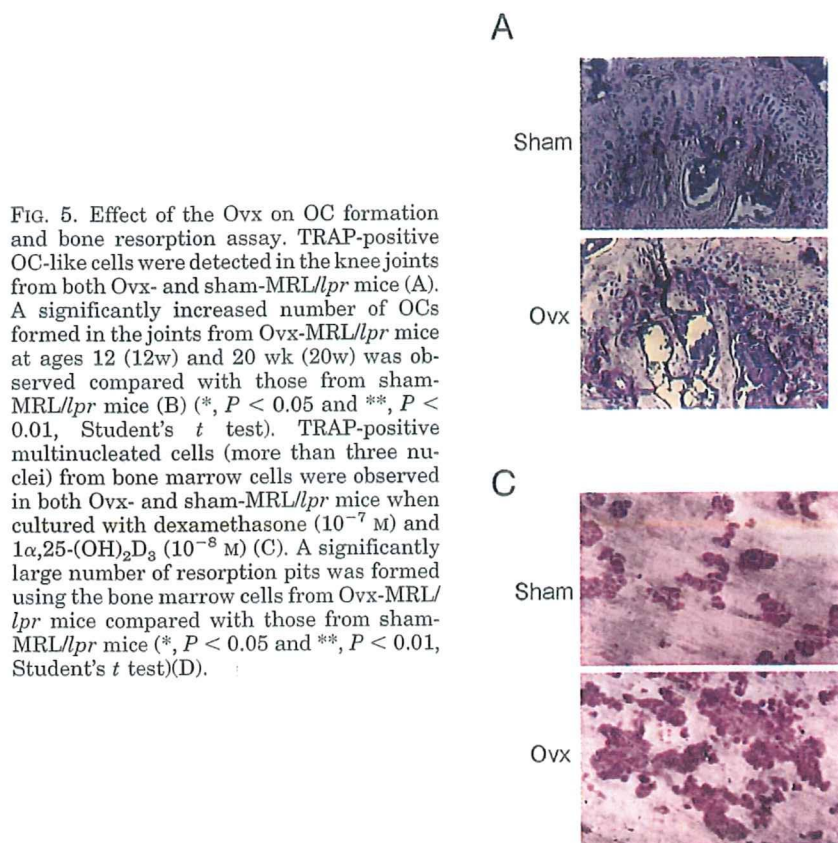
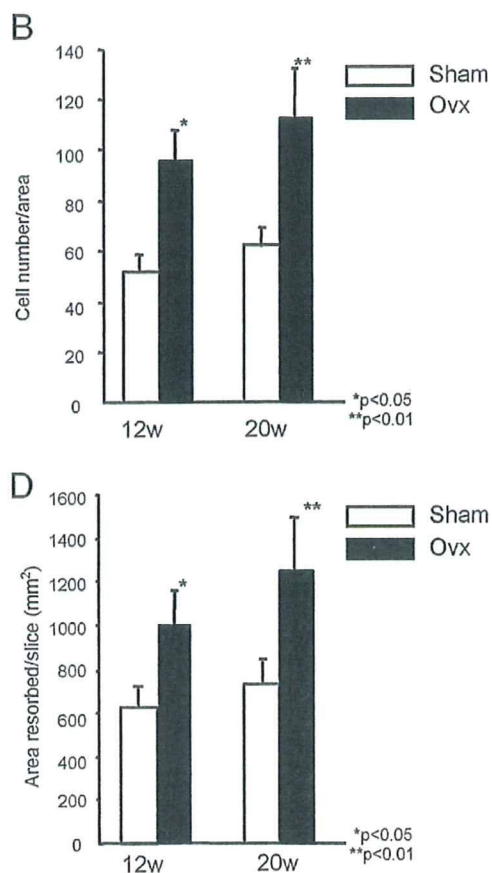


FIG. 5. Effect of the Ovx on OC formation and bone resorption assay. TRAP-positive OC-like cells were detected in the knee joints from both Ovx- and sham-MRL/lpr mice (A). A significantly increased number of OCs formed in the joints from Ovx-MRL/lpr mice at ages 12 (12w) and 20 wk (20w) was observed compared with those from sham-MRL/lpr mice (B) (*, $P < 0.05$ and **, $P < 0.01$, Student's *t* test). TRAP-positive multinucleated cells (more than three nuclei) from bone marrow cells were observed in both Ovx- and sham-MRL/lpr mice when cultured with dexamethasone (10^{-7} M) and $1\alpha,25\text{-(OH)}_2\text{D}_3$ (10^{-8} M) (C). A significantly large number of resorption pits was formed using the bone marrow cells from Ovx-MRL/lpr mice compared with those from sham-MRL/lpr mice (*, $P < 0.05$ and **, $P < 0.01$, Student's *t* test)(D).



ministration. A previous report has shown that treatment with low doses of β -estradiol exerts a suppressive effect on both development of collagen arthritis as well as T cell-dependent immune reactivity toward type II collagen (35). It was also reported that an estrogen deficiency stimulates B cell development (36) and autoantibody production (36–38), and its increase by an estrogen deficiency has been mediated by cytokines such as IL-6, IFN- γ , and TNF- α (39–41). A recent report has demonstrated that estrogen deficiency induces bone loss by increasing T cell proliferation through IFN- γ -induced class II transactivator (42).

Bone resorption is regulated by the immune system, in which T cell expression of RANKL, which is essential for osteoclastogenesis, may contribute to pathological conditions, such as RA (43). However, it remains unclear whether activated T cells maintain bone homeostasis by counterbalancing the action of RANKL. In this study, a significant increase of CD4⁺ T cells bearing RANKL in the spleen and inguinal LN from Ovx-MRL/lpr mice was observed. Moreover, we detected an enhanced RANKL mRNA expression and RANKL⁺ infiltrating cells in synovium from Ovx-MRL/lpr mice. In contrast, an impaired OPG concentration was found in synovial fluid, in addition to a decreased OPG mRNA in synovium of Ovx-MRL/lpr mice. Although molecular mechanisms demonstrating that the appearance of increased RANKL expression in T cells and synovium is related to the development of the autoimmune response are obscure, a role for RANKL in bone resorption in RA is suggested by the identification of RANKL mRNA and protein in

cultured synovial fibroblasts from patients with RA and in CD4⁺ and CD8⁺ T cells in RA synovial tissues (10). Additional evidence that RANKL plays a critical role in the pathogenesis of bone destruction in inflammatory arthritis comes from studies in the rat adjuvant arthritis model (44, 45). Moreover, it has been recently demonstrated that up-regulation of RANKL on bone marrow cells is an important determinant of increased bone resorption induced by estrogen deficiency (46). Treatment with OPG also prevented OC accumulation, whereas destruction of bone in untreated arthritic animals was accompanied by the accumulation of large numbers of TRAP⁺ OC-like cells (47). OPG treatment in an animal model of arthritis that is dependent on T cell activation has the potential for blocking not only the effects of RANKL on OC differentiation and activation but also the influence of RANKL on T cell-DC interactions (20).

OCs have a crucial role in the local bone destruction that occurs in association with chronic inflammatory diseases (48). Diseases such as RA have been associated with the accumulation of TNF- α and/or other proinflammatory cytokines such as IL-1 and IL-6, which likely mediate local bone destruction by stimulating OC activity (49). The results in the present study demonstrated an increased gene expression of cytokines including IL-1 β , TNF- α , IL-6, IFN- γ , β -actin, and IRF-1 in the synovium from Ovx-MRL/lpr mice. In addition, an elevated gene expressions of MMP-3 and MMP-9 mRNA were observed in the synovium. Moreover, we found a significant increase in number of OCs, and bone resorption pits formed in Ovx-MRL/lpr mice. It has been shown that estro-

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

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