the cytoplasm by D-galactosamine plus LPS treatment was 5000–6000 AFC nM/h, this activity is corresponding to the activity by 10^{-6}–10^{-7} M of lactoferrin (calculated by Fig. 3(b)). This amount (concentration) is enough to activate procaspase-3 in vitro. Sufficient amounts of procaspase-3 are located in the normal liver cytoplasm, but caspase-3 was not present in the normal cytoplasm. As a model experiment, the caspase-3 activity (DEVD-AFC cleaving activity) in a normal liver cytoplasm preparation was found to be strongly enhanced by the addition of recombinant lactoferrin in vitro. We reported in our previous papers that the caspase-3 activity in the normal liver cytoplasm was enhanced by the addition of digitonin extracts of lysosomes in vitro [5,6]. The activities of other known caspases [6,8,11,13], which are the proteolytic signal transduction caspases, were not changed by the D-galactosamine treatment (data not shown). When a suitable death signal was added, the corresponding activating protein was translocated from the different organelles into the cytoplasm, resulting in increases in caspase-3 mediated apoptosis, in general [1]. The releasing mechanism of lysosomal lactoferrin by D-galactosamine treatment is not known. Quintero et al. reported that prostaglandin E1 protection against apoptosis induced by D-galactosamine is not related to the modulation of intracellular free radical production in primary culture of rat hepatocyte [19]. However, we have not any direct evidences on the releasing mechanisms of lysosomal lactoferrin into cytoplasm by D-galactosamine induced hepatocyte apoptosis. Fujita et al. reported that lactoferrin stimulated the apoptosis of azoxymethane-induced tumors and the elevation of active forms of caspases-3 and 8. But little is known about the mechanisms at the molecular level. The problem of this report is that the lactoferrin was administrated perorally with diet, it is difficult to consider that the 72-kDa lactoferrin is effectively absorbed from intestine [20]. The caspase-3 activity was dramatically elevated in the cytoplasm of D-galactosamine induced apoptotic hepatocytes and administration of epigallo-catechin gallate which was strong inhibitor for caspase-3 activity suppressed the elevation of caspase-3 activity in the cytoplasm and protected the hepatocyte apoptosis [11]. Our proposed new apoptotic cascade mediated by lysosomal lactoferrin is illustrated schematically in Fig. 5.

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Catechin derivatives: Specific inhibitor for caspases-3, 7 and 2, and the prevention of apoptosis at the cell and animal levels

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Abstract Tea-catechin derivatives are shown to inhibit activities of caspases-3, 2 and 7 in vitro, and prevented experimental apoptosis at the cell and animal levels. Epigallo-catechin-gallate showed the strongest inhibition at 1×10^{-7} M to these caspases, but cysteine cathepsins and caspase-8 were not inhibited. Caspase-3 inhibition showed a 2nd-order allosteric-type, but the inhibition of caspases-2 and 7 showed a non-competitive-type. The apoptosis-test using cultured HeLa cells was inhibited by these catechins. In rat hepatocytes, apoptosis was induced by D-galactosamine in vivo. In this case, caspase-3 activity in the cytoplasm, the serum aminotransferases and dUTP nick formation detected by TUNNEL-staining were effects, and these elevations were suppressed by administration of catechin.

Keywords: Catechin; Caspase-3; Apoptosis; D-Galactosamine; Serum aminotransferase

1. Introduction

Various pharmacological functions of tea-catechin derivatives have been extensively studied in recent years. Their anti-oxidant effects are well established; in addition, the possibility for prevention of oncogenesis by tea-catechins from the aspect of epidemiological statistics has been advocated. However, no reasonable explanation exists for the prevention of oncogenesis at the molecular level (see Section 4). The direct effect of tea-catechins on specific caspases with respect to apoptosis has not yet been reported. The synthetic inhibitors of substrate analogues for caspases have been reported; however, natural inhibitors have not been identified. Allosteric inhibition of caspase-3 by synthetic inhibitors was reported by Hardy et al., therefore the tertiary structures of caspases are flexible (see Section 4) [11]. We have previously shown that some tea-catechin derivatives strongly inhibited caspases-3, 2 and 7, in vitro and in vivo [1,2,5–9].

The inhibition of cultured HeLa cell apoptosis test, which is reported by Wells et al., was studied [4]. Liver injury induced by D-galactosamine with lipopolysaccharide (LPS) in vivo is well characterized to induce hepatocyte apoptosis within the pathological field, assessed by TUNNEL-staining and DNA fragmentation [1–4]. The activity of caspase-3 in the liver cytoplasm was significantly elevated, and aspartate (AST) and alanine (ALT) aminotransferases in the serum were also significantly elevated in the D-galactosamine induced apoptotic liver. These increases were suppressed by epigallo-catechin-gallate (EGCG) in vivo. EGCG is the main component of green tea. The specific inhibition of activities of caspases-3, 2 and 7 by tea-catechin derivatives in vitro and the prevention of liver cell apoptosis in vivo are reported in this paper.

2. Materials and methods

2.1. Materials

Recombinant human caspases-3, 7, 8 and 2 were purchased from Bio-Vision Co. Catechin derivatives were purchased from Wako Co. Cathepsin B and L were purchased from Sigma.

2.2. Methods

2.2.1. Inhibition assays of caspases-3, 7, 2 and 8 activities by catechin derivatives. An established method for the assay of activities of caspase-3 and caspase-7 was used [9], using the recombinant pure caspases and DEVD-AFC as the substrate. Ac-IETD-MCA was used for caspase-8 and AC-VDVAD-MCA was used for caspase-2. Enzyme activity was expressed as the released AFC (or MCA) formed nM/h/mg protein.

2.2.2. Cell-free apoptosis test using cultured HeLa cell S-100. The apoptosis assay system reported by Wells et al. is composed of cultured HeLa cell cytoplasm S-100 (4 mg protein/ml), cytochrome *c* (80 μM) and Ac-DEVD-MCA (40 μM) as the substrate for formed caspase-3 [12]. Preparation of S-100 from cultured HeLa cells was followed using the method described by Wells and Nguyen [12]. Following incubation at 37 °C for 40 min, the released fluorescent MCA in the S-100 fraction was assayed as formed caspase-3 from procaspase-3 in the S-100. Caspase-3 activity without addition of cytochrome *c* was used as the negative control.

2.2.3. Administration method of D-galactosamine and tea-catechin derivatives in rats. Liver apoptosis was induced according to Muntane's method, by intraperitoneal injection of D-galactosamine [3,4]. A single dose of D-galactosamine was administered intraperitoneally

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Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; EGCG, epigallo-catechin gallate; ECG, epi-catechin gallate; CG, catechin gallate; EC, epi-catechin; EGC, epigallo-catechin; C, catechin; GC, gallo-catechin; G, gallate; LPS, lipopolysaccharide; TdT, terminal transferase; MCA, methyl coumaryl amide

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(0.5 g/kg), and rats were sacrificed 12 h after the injection. Two doses of EGCG with 50 μg/kg of LPS were administered intraperitoneally at 1 h before and after the D-galactosamine administration. EGCG was further administered twice at 3-h intervals.

2.2.4. Preparation of liver cytoplasm for assay of caspase-3 activity. Liver cytoplasm fraction for caspase-3 activity assay was prepared by sequential centrifugation method for cell organelle separation according to a method described by Fleisher and Kervina [16].

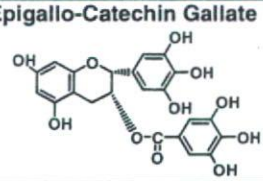
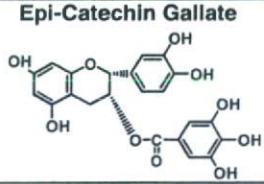
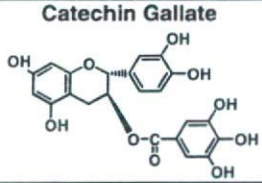
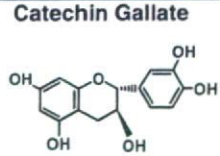
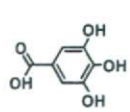
2.2.5. TdT-mediated dUTP nick end labeling (TUNNEL) assay. Apoptotic cells were detected in sections using the in situ Apoptosis Kit (Takara Kyoto, Japan). Frozen sections of liver tissues were fixed in 3% paraformaldehyde, incubated with protease K (20 μg/ml) for 10 min, and then presoaked in terminal transferase (TdT) buffer (0.5 μM/L cacodylate, 1 μM/L CoCl, 0.5 μM/L dithiothreitol, 0.05% bovine serum albumin, and 0.15 M/L NaCl) for 10 min. Sections were incubated for 1 h at 37 °C in 25 ml of TdT solution, containing 1× terminal transferase buffer, 0.5 nM of biotin-dUTP, and 10 U of TdT. After the TdT reaction, sections were soaked in TdT blocking buffer (300 nM/L NaCl and 30 mM/L tri-sodium citrate-2-hydrate), incubated with HRP-conjugated streptavidin for 30 min at room temperature, and developed for 10 min in phosphate-buffered citrate (pH 5.8) containing 0.6 mg/ml DAB. Nuclei were counterstained with hematoxylin.

3. Results

3.1. Inhibition of caspase-3 activity by various catechin derivatives in vitro

Caspase-3 plays a central role as an executive enzyme of apoptosis in the final step of various apoptotic cascades [5–9].

Table 1
Comparison of inhibition of caspase-3 activity in vitro and the apoptosis test using cultured HeLa cells by tea-catechin derivatives

Catechin Derivatives	*in vitro* 50% Inhibition of caspase-3	HeLa cell apoptosis test 50% inhibition of apoptosis test
Epigallo-Catechin Gallate 	1×10^{-8} M	1×10^{-6} M
Epi-Catechin Gallate 	1×10^{-7} M	1×10^{-4} M
Catechin Gallate 	1×10^{-6} M	5×10^{-4} M
Epigallo-Catechin 	1×10^{-6} M	5×10^{-4} M
Epi-Catechin 	1×10^{-6} M	5×10^{-4} M
Catechin Gallate 	Gallo-Catechin 	Gallic Acid 	No Inhibitions by 1×10^{-4} M

The left-hand column shows the concentrations of catechin derivatives inducing 50% inhibition of caspase-3 activity in vitro. The right-hand column shows the 50% inhibition of the apoptosis test units of cultured HeLa cells. The assay methods are described in the text [10,11]. The 50% inhibition concentrations of various catechin derivatives are illustrated ($n = 3$, the mean ± S.E.M. with $^{*}P < 0.01$).

Caspase-3 activity was completely inhibited by EGCG at 1×10^{-7} M and was inhibited to 50% at 1×10^{-8} M in vitro. Epi-catechin gallate (ECG) showed 50% inhibition at 1×10^{-7}M, and catechin gallate (CG), epi-catechin (EC) and epigallo-catechin (EGC) had induced inhibition at 1×10^{-6} M. Catechin (C), gallo-catechin (GC) and gallate (G) showed no inhibition as Table 1 shows. The stereo-binding form of –OH to the catechin-ring should be an epi-structure to display inhibitory activity. The presence of either component, catechin gallate (CG) and/or epi-form catechin (EC), is essential.

Relationship of velocity and substrate concentration of caspase-3 in the presence of EGCG showed a typical sigmoidal curve and the Lineweaver–Burk relationship did not give a straight line, but showed a logarithmic curve. When the abscissa was taken as $1/[S]^2$, the logarithmic curve changed to a straight line (Fig. 1A). The inhibition kinetics of these catechin derivatives appear to be a 2nd-order sigmoidal allosteric inhibition as follows:

$$1/v = Km/V(1/[S]^2) + 1/V.$$

The other four effective catechin derivatives, such as ECG, CG, EC and EGC, also showed the same type of allosteric inhibition to caspase-3 as that by EGCG (figures are abbreviated).

The binding site of the catechins appeared to be different from the substrate-binding site. The allosteric nature of caspase-3 using synthetic inhibitors was reported by Hardy et al. [11] (see Section 4). The molecular weight of caspase-3 did not appear to change in the presence of EGCG and/or substrate using Superdex G-75. Therefore, polymerization or depolymerization was not observed using these allosteric inhibitors (data not shown).

3.2. Inhibitions of activities of caspases-7 and 2 activities by EGCG in vitro

Caspases-7 and 2 are also known to participate in various apoptosis cascades. The activities of caspases-7 and 2 were also strongly inhibited by EGCG, and the 50% activities were inhibited at 1×10^{-6} M. However, the mode of inhibitions of caspases-7 and 2 were different from that of caspase-3. The V_{max} decreased in the presence of EGCG and the Lineweaver–Burk relationship showed a non-competitive type inhibition (Fig. 1B and C). The binding site to EGCG is the same as the substrate-binding site or located near the active site. Caspase-8, cathepsins B and L, which are the same cysteine proteases, were not inhibited at 1×10^{-5} M of EGCG. Therefore, the inhibitions of caspases are not due to an attack to the active site –SH of these enzymes by the scavenger effect of catechins.

3.3. Inhibition of caspase-3 in HeLa cell apoptosis test induced by cytochrome c by EGCG

Wells et al. developed a cell-free apoptosis test using cultured HeLa cells [12]. The S-100 prepared from cultured HeLa cell cytoplasm contains sufficient amounts of procaspase-3 and the activating enzyme system except cytochrome *c*. Caspase-3 activity in the S-100 increased following the addition of cytochrome *c*, as shown in Fig. 2. The 70% of the apoptosis unit was inhibited by EGCG at a concentration of 1×10^{-5} M. The strengths of suppression by the various catechin derivatives were in the same order as the inhibitions of caspase-3 activity in vitro, as shown in Table 1.

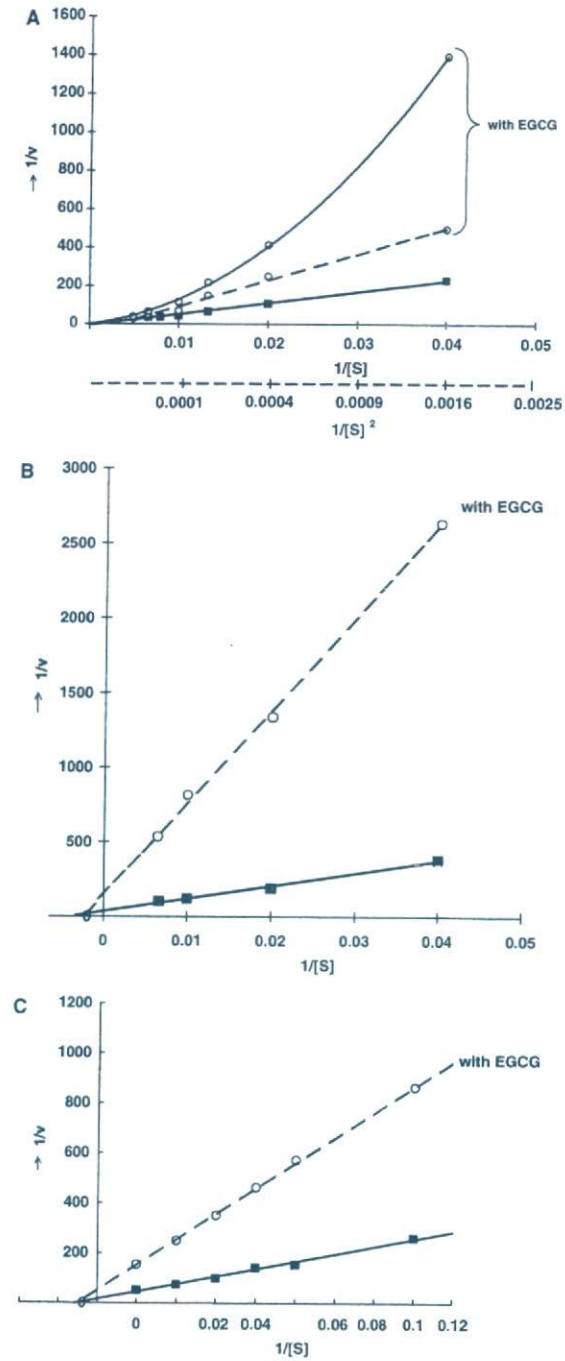


Fig. 1. Mode of inhibitions of caspase-3, 2 and 7 by EGCG in Lineweaver–Burk relationship. (A) Caspase-3 inhibition by 1×10^{-7} M of EGCG. The $1/v$ values to the $1/[S]$ in the presence of EGCG are expressed as open circles with a thin solid line (○–○). The $1/v$ values to the $1/[S]$ in the absence of EGCG are illustrated as closed squares with a solid line. The $1/v$ values to the $1/[S]^2$ illustration in the presence of EGCG are expressed as open circles with a broken line (○---○). (B) and (C) Caspase-2 or 7 inhibition by 1×10^{-6} M of EGCG. The activities in the absence of EGCG are illustrated as solid line with a solid line and the activities in the presence of EGCG are illustrated as open circles with a broken line. (B) shows caspase-2 inhibition by EGCG; the Lineweaver–Burk relationship. (C) shows caspase-7 inhibition by EGCG. All symbols and lines are the same as those in (B).

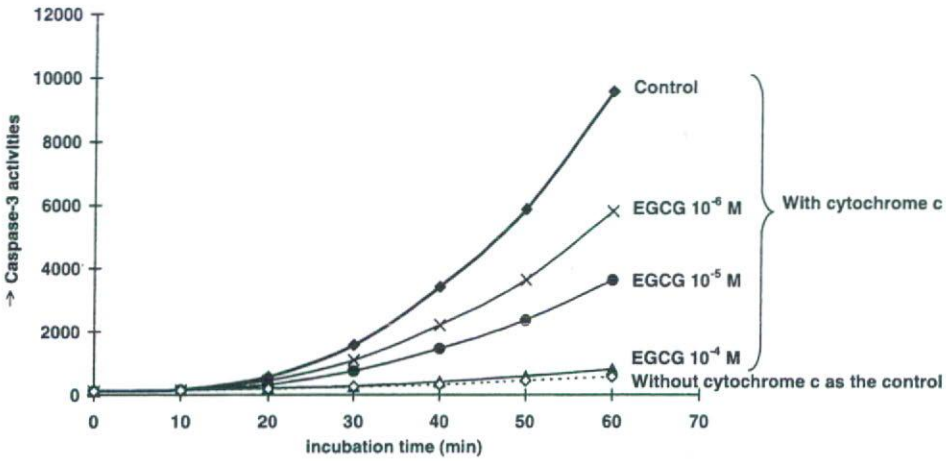


Fig. 2. Inhibition of caspase-3 activities in apoptotic test using cultured HeLa cells induced by cytochrome *c* by EGCG. Caspase-3 inhibition in S-100 of cultured HeLa cells by various catechin derivatives was assayed using Nguyen and Wells' apoptosis test as shown in Table 1, right panel [12]. The inhibitory activities by various catechin derivatives are compared as 50% inhibition concentrations as shown in Table 1, in left panel. The catechin derivatives added were expressed as the final concentrations in the S-100 fraction. All data are the means ± S.E.M. ($n = 5$) with $^{*}P < 0.01$.

3.4. Liver apoptosis induced by D-galactosamine plus LPS, and its prevention by EGCG in vivo

Sufficient amounts of procaspase-3 are present and active caspase-3 is not present in the normal hepatocyte cytoplasm. However, procaspase-3 in the cytoplasm is activated to form active caspase-3 by the effective apoptotic signal. It is well known within the pathological field that hepatocyte injury induced by D-galactosamine results in hepatocyte apoptosis, as assessed by the TUNNEL-staining and the DNA ladder formation [3,4,10].

(1) Elevations of liver caspase-3 activity and serum aminotransferases in D-galactosamine induced hepatocyte apoptosis, but were prevented by cotreatment with EGCG, as shown in Table 2. The both elevations were prevented by cotreatment with EGCG in a dose-dependent manner, and treatments with 50 mg/head EGCG suppressed the activity to the normal level. Furthermore, the macroscopic liver profile was protected and resembled to normal level.

However, the mechanism of procaspase-3 activation cascade induced by D-galactosamine remains unknown (see Section 4).

(2) TUNNEL-staining method, which is the most established DNA nick formation in the nucleus, was examined in these livers. As shown in Fig. 3, the significant nick staining of nuclear DNA was observed in the livers treated with D-galactosamine, while nick formations was significantly suppressed by cotreatment with EGCG. These data show that D-galactosamine induced liver injury resulted in caspase-3 mediated apoptosis and the apoptosis was significantly suppressed by EGCG administration.

(3) Increased activities of AST and ALT in the serum by D-galactosamine administration, which are the established marker for hepatocyte injury, were also completely suppressed by cotreatment with EGCG dose-dependently as shown in Table 2. EGCG showed an effective protecting effect for the liver injury mediated by caspase-3.

4. Discussion

There are several papers on cancer prevention by tea-catechin derivatives, which appear to contradict our own data.

Table 2
Elevation of caspase-3 activities in rat liver cytoplasm in vivo and the activities of AST and ALT in the serum following D-galactosamine administration, and the preventions by EGCG treatment in vivo

	Caspase-3 activities in liver (AFC nM/mg/h)	Aminotransferases in serum (IU/l)	
		AST	ALT
Control LPS	<100	<37.8	<5.8
D-GalN	1000.0	450.0	300.0
D-GalN + LPS	5500.0	5229.3	1438.3
D-GalN + LPS + EGCG 10 mg	3500.0	–	–
D-GalN + LPS + EGCG 30 mg	300.0	320.5	114.0
D-GalN + LPS + EGCG 50 mg	100.0	<100	<100

0.5 g/kg D-galactosamine with 50 μg/kg LPS was administered once intraperitoneally. The D-galactosamine administration method and the preparations of the liver cytoplasm for the caspase-3 assay are described in Section 2.2. The injection doses of EGCG are mg/head. Elevation of caspase-3 activities in the liver cytoplasm in D-galactosamine-induced apoptosis and the preventions by EGCG are in the left columns. The dose-dependent suppressions of caspase-3 activities by EGCG administration are represented in the left columns. All data represent the means ± S.E.M. ($n = 5$) with $^{*}P < 0.01$.

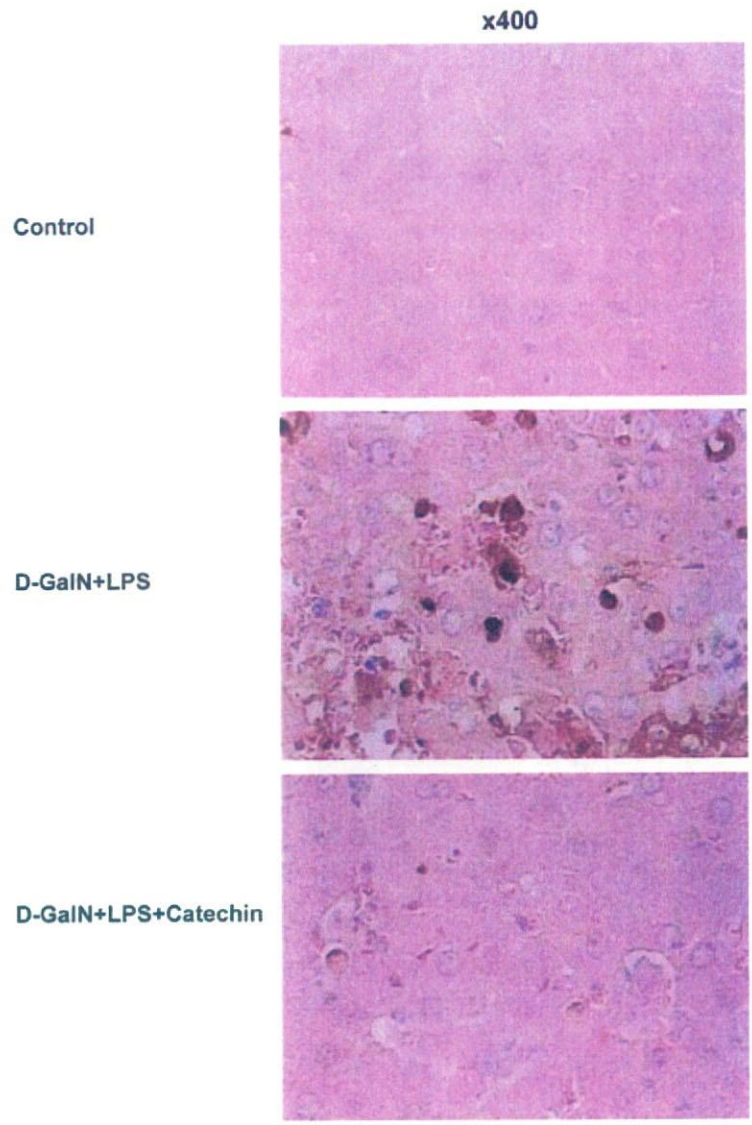


Fig. 3. Hepatocyte apoptosis images using TUNNEL-staining induced by D-galactosamine plus LPS and its prevention by EGCG cotreatment. The staining method is described in Section 2.2. These images are 400× magnification. Top image shows the control liver, the middle image shows liver administrated D-galactosamine plus LPS and the suppression profile by EGCG is shown in the bottom image.

However, this is completely different phenomenon from the following reasons; the reported effective concentration of catechin for cancer prevention is very high 10^{-3}–10^{-4} M [13], these concentrations are not physiological and appear to be toxic concentration. On the other hand, inhibition of caspase-3 by catechins was 10^{-6}–10^{-7} M in vitro and in vivo. Furthermore, these papers do not mention on the relationship between cancer cell death and apoptosis mediated by caspases [13–15]. Some papers reported that catechin stimulates release of TNF-α and enhances effect of anticancer drugs in vivo. While there is data demonstrating the prevention of oncogenesis in vivo, there is no research at the molecular level [14,15].

There are two possible mechanisms by which catechin suppresses hepatocyte apoptosis induced by D-galactosamine administration. One is due to direct inhibition of caspase-3 activity and the other is due to elimination of O_2^-, which is produced by D-galactosamine-protein binding through Maillard reaction. Both mechanisms are likely.

Caspase-3 is constructed from a heterotetramer, which is composed of two pairs of heterodimers. Each unit is composed of a long chain and a short chain. The substrate-binding site is located in the long chains. The interaction between the long chain and short chain and also the unit-to-unit interactions are susceptible to allosteric effectors. For example, it has been reported by Hardy et al. [11] using synthetic allosteric inhibitors that the inhibitor-binding site of the caspase-3 molecule is different from the substrate binding site. They also reported that the –SH of these inhibitors can form a disulfide bond with the cysteine-SH at amino acid 290th of the enzyme, which is different from the active site cysteine in the long chain. The practical conformational change by EGCG will be made clear using X-ray co-crystallography.

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Immunology and Infectious Diseases

Analysis of *in Vivo* Role of α-Fodrin Autoantigen in Primary Sjögren's Syndrome

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The α-fodrin N-terminal portion (AFN) autoantigen mediates *in vivo* immunoregulation of autoimmune responses in primary Sjögren's syndrome (SS). We further examined this process and found that cleavage products of AFN were frequently detected in the salivary gland duct cells of SS patients. In *in vitro* studies using human salivary gland HSY cells, anti-Fas-induced apoptosis resulted in specific cleavage of α-fodrin into the 120-kd fragment, in association of α-fodrin with μ-calpain, and activation of caspase 3. Significant proliferative responses against AFN autoantigen were observed in the peripheral blood mononuclear cells (PBMCs) from SS patients with higher pathological score (grade 4) and with short duration from onset (within 5 years). *In vivo* roles of AFN peptides were investigated using PBMCs from patients with SS, systemic lupus erythematosus, and rheumatoid arthritis. Significant proliferative T-cell responses of PBMCs to AFN peptide were detected in SS but not in systemic lupus erythematosus or rheumatoid arthritis. AFN peptide induced Th1-immune responses and accelerated down-regulation of Fas-mediated T-cell apoptosis in SS. Our data further elucidate the *in vivo* role of AFN autoantigen on the development of SS and suggest that the AFN autoantigen is a novel participant in peripheral tolerance. *(Am J Pathol 2005, 167:1051–1059)*

Organ-specific autoimmune diseases are characterized by tissue destruction and functional decline due to autoreactive T cells that escape self-tolerance.[1,2] Primary Sjögren's syndrome (SS) 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.[3–5] SS is a T-cell-mediated autoimmune disease, and autoreactive T cells bearing CD4 molecule may recognize unknown self antigen-triggering autoimmunity in the salivary and lacrimal glands, leading to clinical symptoms of dryness of the mouth and eyes (sicca syndrome).[6,7] Accumulated evidence suggest an important role of apoptosis in disease pathogenesis of SS.[8] Previously we have identified a 120-kd α-fodrin autoantigen in the pathogenesis of primary SS,[9] but the role of autoantigen that render *in vivo* immunoregulation remains unclear.

Although an important role for T cells on the development of organ-specific autoimmune disease has been argued, it is not known whether disease is initiated by a restrained inflammatory reaction to an organ-specific autoantigen. Autoreactive T cells generally respond to a limited number of immunodominant epitopes in self-antigenic proteins including myelin basic protein, thyroglobulin, and glutamic acid decarboxylase.[10–12] α-Fodrin is a ubiquitous, heterodimeric calmodulin-binding protein[13] found to be cleaved by calcium-activated protease (calpain) in apoptotic T cells, and by calpain and caspase family cysteine proteases[14] in anti-Fas-stimulated Jurkat cells and/or neuronal apoptosis.[15–17] Previous reports have demonstrated evidence that caspase 3 is required for α-fodrin cleavage during apoptosis.[18–20] In Jurkat cells, caspase 3-like proteases have been reported to cleave α-fodrin and poly (ADP-ribose) polymerase but with differential sensitivity to the caspase 3 inhibitor, DEVD-fmk.[20] In neuroblastoma cells, treatment with staurosporin induced cleavage of α-fodrin at both caspase 3 and calpain cleavage sites.[21] Therefore, we speculate that an increase in enzymatic activity of apoptotic proteases is involved in the progression of α-fodrin proteolysis during apoptosis of human salivary gland cells. In this study, we analyzed Fas-mediated apoptosis in SS

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salivary glands, and the *in vivo* role of the autoantigen for T-cell response, cytokine production, and peripheral tolerance.

Materials and Methods

Patients with Autoimmune Diseases

Peripheral blood samples from 18 patients with primary SS, 6 systemic lupus erythematosus (SLE), and 5 rheumatoid arthritis (RA), and from age-matched healthy donors (n = 18) were obtained from the Tokushima University Hospital, Tokushima, Japan. SLE and RA patients were diagnosed based on American College of Rheumatology criteria.[22,23] All patients with SS were female, had documented xerostomia and keratoconjunctivitis sicca, and fulfilled San Diego criteria for the diagnosis of SS.[3] Patients with secondary SS were carefully excluded. All patients with SS had focus scores of greater than 2 in their lip biopsy and all tested positive for autoantibodies against Ro, and 15 of 18 SS patients had autoantibodies against 120-kd α-fodrin by Western blotting. Analysis was performed under the certification of the ethics board of Tokushima University Hospital.

Immunohistology

Immunohistology was performed on freshly frozen sections (4 μm in thickness) by the biotin-avidin immunoperoxidase method using ABC reagent (Vector Laboratories, Burlingame, CA). Briefly, freshly frozen sections were fixed in acetone for 10 minutes, rinsed in phosphate-buffered saline (PBS, pH 7.2), and incubated with an appropriate blocking reagent (Vector Laboratories) for 20 minutes. They were incubated for 1 hour with biotinylated mouse monoclonal antibodies (mAbs) to CD4, CD8, L26(CD20) (BD Bioscience, San Jose, CA), and to Fas and FasL (BD PharMingen, San Diego, CA). To detect the cleavage product of α-fodrin, polyclonal rabbit Abs raised against the synthetic peptide to the purified 120-kd antigen corresponding to the identified 20 amino acid residues (RQKLEDSYRFQFFQRDAEEL) were developed and used.[9] Isotype-matched sera were used as controls, respectively.

Production of Recombinant α-Fodrin

Recombinant α-fodrin N-terminus (AFN) protein (JS-1), the cDNA encoding human α-fodrin (JS-1:1,1784 bp)[9] was constructed by inserting cDNA into the *Eco*RI site of pGEX-2T. Glutathione *S*-transferase fusion protein was expressed and purified using a glutathione *S*-transferase gene fusion system (Amersham Bioscience, Piscataway, NJ).

Synthetic Peptides

AFN peptides identical to JS-1 region were synthesized using tent-butoxycarbonyl chemistry on a model 430A peptide synthesizer (Applied Biosystems, Foster City, CA). A total of 45 synthetic peptides that were designed to be 20-amino acid residues in length, overlapping by five-amino acid residues were generated. As control peptide, laminin fragment peptide 929-933 (Sigma Chemical Co., St. Louis, MO) was used.

Proliferative T-Cell Response

Freshly isolated peripheral blood mononuclear cells (PBMCs) from SS patients and age-matched controls were assayed. When necessary, isolated $CD4^+$ and $CD8^+$ T cells from PBMCs using magnetic beads (Dynal, Oslo, Norway) were assayed. Single cell suspensions were cultured in 96-well flat-bottom microtiter plates (5×10^5 cells/well) in RPMI 1640 containing 10% fetal calf serum, penicillin/streptomycin, and β-mercaptoethanol. Cells were cultured with 10 μg/ml JS-1 protein, 10 μg/ml AFN peptide, and 2.0 μg/ml Con A (EY Laboratories, San Mateo, CA). To confirm the immunoreactivity with the AFN protein (JS-1), 2×10^5 $CD4^+$ and $CD8^+$ T cells from PBMCs of SS patients and controls were co-cultured with irradiated T-cell-depleted PBMCs as antigen-presenting cells, and stimulated with 0 to 20 μg/ml JS-1 for 72 hours. During the last 8 hours of the 72-hour culture period, 1 μCi of [^{3}H]thymidine was added per well, and the incorporated radioactivity was determined using an automated β liquid scintillation counter.

Flow Cytometric Analysis

For analysis of intracellular cytokines, monensin (Wako Pure Chemical, Osaka, Japan) was added at 2 μmol/L to isolated PBMCs (10^6/ml), and 2 hours later the cells were collected, labeled with anti-CD4-PE (BD PharMingen), fixed with 4% paraformaldehyde for 10 minutes at 4°C, and then permeabilized with 0.1% saponin in PBS at room temperature for 10 minutes. Cells were incubated with anti-interleukin (IL)-2-fluorescein isothiocyanate (FITC) (8 μg/ml; BD PharMingen), anti-IL-4-FITC (5 μg/ml; BD PharMingen), and anti-interferon (IFN)-γ-FITC (1 μg/ml; BD PharMingen), respectively, and analyzed on a EPICS flow cytometer (Beckman Coulter, Miami, FL). For analysis of Fas and FasL expression, isolated PBMCs from SS patients when pulsed with AFN peptide (10 μg/ml) were assayed by flow cytometry gated on $CD4^+$ T cells, using anti-Fas and anti-FasL mAb (BD PharMingen). Mean fluorescence intensity was calculated using the fluorescence intensity of staining for mAbs to Fas or FasL and isotype-matched control measured by flow cytometry. Apoptotic cells were detected by flow cytometry with an EPICS (Beckman Coulter) using the Annexin V-FITC apoptosis detection kit (Genzyme, Cambridge, MA).

Cell Culture and Induction of Apoptosis

Human parotid salivary gland HSY cells[24] were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere

of 5% CO_2 in air at 37°C. The cells were maintained in a logarithmic growth phase by routine passage every 2 to 3 days. Apoptosis was induced in HSY cells by anti-Fas mAb (clone CH-11; Medical and Biological Laboratories. Co., Ltd., Nagoya, Japan).

Western Blot Analysis

For detection of a cleavage product of α-fodrin, Western blot analysis was performed with anti-α-fodrin mAb (Affiniti, Mamhead, UK). To detect the apoptotic proteases *in vitro*, Western blot analysis was performed using mouse mAbs to μ-calpain (clone 9; Chemicon Int., Temecula, CA) specific for catalytic subunit (80 kd), calpastatin (clone 1F7E3D10; Calbiochem, San Diego, CA) specific for amino acids 543 to 673 encoding domain IV (150, 125, 90, and 70 kd), and caspase 3 (3G2; Transduction Laboratories, Lexington, KY) specific for amino acids 28 to 44 encoding large subunit (17/19 kd). The cells were homogenized in 20 mmol/L Tris-HCl buffer (pH 7.4) containing 5 mmol/L ethylene diamine tetraacetic acid, 10 μl/ml protease inhibitor cocktail (Sigma Chemical Co.) and 0.2% Triton X-100. After centrifugation for 20 minutes at 12,000 × *g* at 4°C, supernatant was extracted and used for cytoplasmic protein. Pellets were homogenized in 20 mmol/L Tris-HCl buffer containing 2% Triton X-100. Protein binding was visualized with ECL Western blotting reagent (Amersham Bioscience). Protease inhibitors included leupeptin, E64, pepstatin (Wako Pure Chemicals), calpain inhibitor peptide (Sigma Chemical Co.), and caspase inhibitors [Ac-YVAD-CHO (ICN, Costa Mesa, CA); Z-VAD-fmk (ICN)].

Sequential Activation of Caspase-Like Proteases

The caspase 3-like activity in anti-Fas mAb-treated HSY cell extracts was determined using fluorescent substrate.[25] Cell lysates were diluted with 0.5 ml of standard buffer, and incubated at 30°C for 30 minutes with 1 μmol/L fluorescent substrate. The specific inhibitor for caspase 3 (Z-DEVD-fmk) was added to the reaction mixture at a concentration of 1 μmol/L. Specific caspase 3-like activity was determined by subtracting the values obtained in the presence of inhibitors. The fluorescent substrate, MOCAc-DEVD (dnp)-NH_2 was custom-synthesized at the Peptide Institute (Osaka, Japan). The fluorescence of the cleaved substrates was determined using a spectrofluorometer set at an excitation wavelength of 328 nm and an emission wavelength of 393 nm.

Cell Transfection

cDNAs for full-length caspase 3 and μ-calpain obtained by polymerase chain reaction were subcloned into the pCRII vector (Invitrogen Co., Carlsbad, CA). All constructs were confirmed by DNA sequencing. For expression experiments, DNA fragments were subcloned into pcDNA3.1 expression vector (Invitrogen Co.). HSY cells were transfected with the pcDNA3.1 expression vectors using the Lipofectamine reagent according to the manufacturer's instruction (Invitrogen Co.). The cells were transfected with the individual plasmid DNA and the total amount of DNA transfected was adjusted to 10 μg with pcDNA3.1 for each 100-mm dish or 3 μg for each 60-mm dish. After a 5-hour incubation with the DNA/lipid mixture, the cells were washed with PBS before replenishing with growth media. The cells were harvested 24 hours after transfection and lysed in Tris-HCl buffer.

Results

Involvement of Apoptotic Cascade in SS Salivary Glands

Immunohistochemical analysis revealed that a majority of infiltrating cells were $CD4^+$, and that a small number of $CD8^+$ cells were present in the SS salivary glands. $L26^+$ B cells were sporadically present in the inflammatory lesions (data not shown). Shown in Figure 1, A and B, are photomicrographs taken from representative data. Immunofluorescence analysis revealed that a large number of infiltrating lymphoid cells bear FasL in SS salivary glands (Figure 1C), and epithelial duct cells stained positively with Fas on their cell surface (Figure 1D). Some acinar cells were stained negligibly positive with both anti-FasL and anti-Fas antibodies, but most acinar cells were negative. Immunofluorescence analysis using polyclonal Ab against synthetic 120-kd α-fodrin[9] demonstrated that a cleavage product of α-fodrin was present in epithelial duct cells of the labial salivary gland biopsies from SS patients, but not in control glands (Figure 1, E and F). Western blot analysis confirmed the same results (Figure 1G), indicating that a cleavage product of 120-kd α-fodrin is present in the diseased glands with SS.

In Vitro *Cleavage of α-Fodrin Induced by Apoptotic Stimuli*

We examined the *in vitro* cleavage of α-fodrin using HSY and Jurkat cells induced by anti-Fas mAb (ranging from 1 to 1000 ng/ml^{-1}) apoptotic stimuli. Anti-Fas (CH-11)-stimulated apoptosis in HSY cells was confirmed by flow cytometric analysis using an apoptosis detection kit as well as in Jurkat cells (Figure 2A). Western blot analysis demonstrated that the 240-kd α-fodrin on apoptotic HSY cells was cleaved into smaller 120-kd fragments in dose-dependent manner of anti-Fas mAb (Figure 2B). We next examined whether α-fodrin cleavage into the 120-kd fragment on apoptotic HSY cells could be blocked by preincubation with specific protease inhibitors. In apoptotic HSY cells, a combination of calpain inhibitor peptide and caspase inhibitor (Z-VAD-fmk) entirely blocked the formation of 120-kd α-fodrin, whereas calpain inhibitor peptide alone could not block 120-kd formation (Figure 2C). Caspase inhibitor alone could block considerably 120-kd formation. Cysteine protease inhibitors (E64), serine pro-

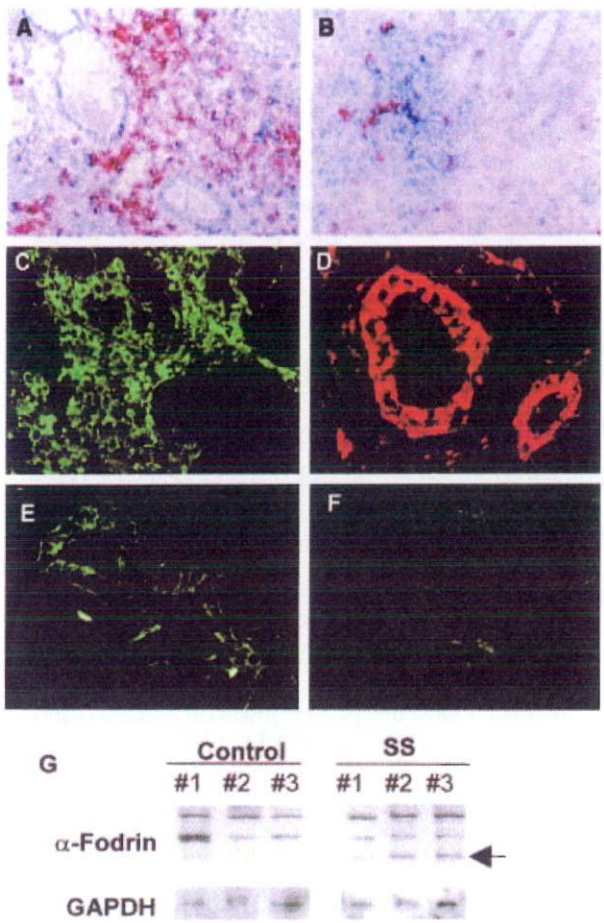

Figure 1. Representative immunohistological features in the labial gland biopsies. A majority of infiltrating cells were $CD4^+$ (**A**), and a small number of $CD8^+$ T cells (**B**) were present in the SS salivary glands. Five samples were examined. Immunofluorescence analysis revealed that the majority of tissue-infiltrating lymphoid cells bear FasL (**C**), and epithelial duct cells stained positively with Fas on their cell surface (**D**) in SS salivary glands. Isotype-matched controls were stained negatively. Six samples for each were examined. A cleavage product of 120-kd α-fodrin was present exclusively in epithelial duct cells of the SS salivary glands (**E**), but not in control salivary glands (**F**). Six samples for each were examined. Detection of a cleavage product of 120-kd α-fodrin in the labial salivary gland biopsies with SS (no. 1, no. 2, and no. 3), but not in control individuals (no. 1, no. 2, and no. 3) on Western blotting (**G**). Eight samples for each were examined.

tease inhibitor (leupeptin), and acidic protease inhibitor (pepstatin) had no effect on 120-kd α-fodrin cleavage.

Calpain and Caspase Mediated α-Fodrin Cleavage

We investigated whether cysteine proteases are involved in α-fodrin cleavage on apoptotic HSY cells. We found a constitutive expression of μ-calpain, and its time-dependent increase in anti-Fas-stimulated HSY cells (Figure 3A). Of note is that abundant calpastatin activity is shown to be constitutively expressed more than calpain expression, and a time-dependent decrease of calpastatin expression was observed in apoptotic HSY cells, not in Jurkat cells (Figure 3A). It can be speculated that μ-calpain activity could be considerably affected by endogenous calpastatin during apoptosis in HSY cells. We con-

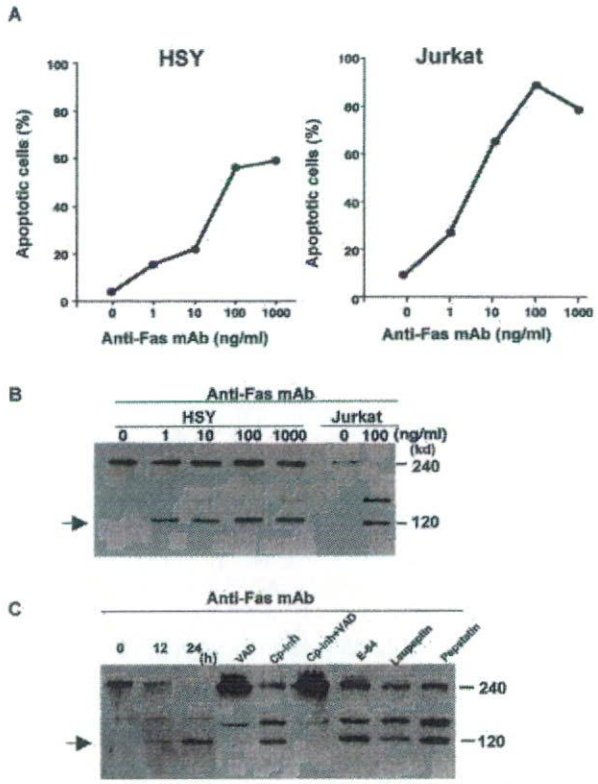

Figure 2. Anti-Fas-induced apoptosis in HSY and Jurkat cells. **A:** The HSY and Jurkat cell apoptosis induced by anti-Fas mAb (CH-11) stimulation was determined by flow cytometry of DNA content of nuclei with PI and annexin V. **B:** Western blot analysis demonstrated 120-kd α-fodrin in apoptotic HSY and Jurkat cells in a dose-dependent manner. **C:** Effects of protease inhibitors on α-fodrin cleavage in apoptotic HSY cells. Proteolytic cleavage of α-fodrin to 120 kd in Fas-stimulated HSY cells is blocked by a combination of a calpain inhibitor peptide and caspase inhibitors (Z-VAD-fmk), but not by E64, leupeptin, and lepstatin. Calpain inhibitor peptide alone could not inhibit the 120-kd α-fodrin formation.

firmed a time-dependent increase in the active form of μ-calpain in apoptotic HSY cells (Figure 3A). Anti-Fas-stimulated HSY cells were positive for mAbs to caspase 3 in association with apoptosis (Figure 3B). Moreover, the caspase 3-like activities in anti-Fas Ab-stimulated HSY cell extracts were determined using fluorescent substrates (Figure 3B).[25] To confirm the role of caspase 3 and μ-calpain proteins in induction of α-fodrin cleavage, full-length caspase 3 and μ-calpain cDNAs were transiently overexpressed in HSY cells, and cleavage product of 120-kd α-fodrin was examined by anti-α-fodrin Ab. Analysis of lysates from caspase 3 and μ-calpain cDNA co-transfected cells with Western blotting revealed a significant increase (approximately fivefold to sevenfold) of 120-kd α-fodrin in the level of expression of caspase 3 or μ-calpain in cells transfected with respective cDNA (Figure 3C).

In Vivo *Role of α-Fodrin in SS Patients*

To confirm the immunoreactivity with the AFN protein (JS-1), $CD4^+$ and $CD8^+$ T cells were isolated from PBMCs of SS patients (n = 3) and controls (n = 2), and were co-cultured with irradiated T-cell-depleted

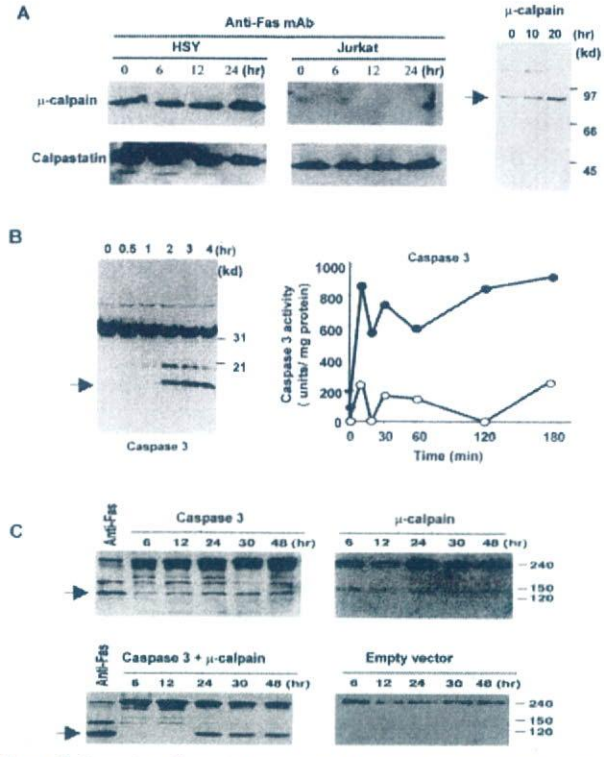

Figure 3. Detection of cysteine proteases in anti-Fas-induced apoptotic HSY cells. Western blot analysis of μ-calpain, and calpastatin in apoptotic HSY and Jurkat cells stimulated with anti-Fas Ab (CH-11). A constitutive expression of μ-calpain, and its time-dependent increase were observed in anti-Fas-stimulated HSY cells. Calpastatin activity is shown to be constitutively expressed,more than calpain expression and a time-dependent decrease of calpastatin expression was observed in apoptotic HSY cells, not in Jurkat cells. **A:** Western blot analysis of the active form of μ-calpain in apoptotic HSY cells stimulated with anti-Fas mAb (CH-11). **B:** Western blot analysis showing a time-dependent increase in caspase 3 and sequential activation of caspase 3-like protease in anti-Fas-induced apoptotic HSY cells. The caspase 3-like activity in the lysates (100 mg protein) (**filled circle**) or in the presence of 50 mmol/L MOCAc-DEVD-NH2 (**open circle**) was determined using fluorescent substrates in apoptotic HSY cells. One unit corresponds to the activity that cleaves 1 pmol of the respective fluorescent substrate at 30°C in 30 minutes. **C:** Detection of cleavage product of 120-kd α-fodrin in co-transfected HSY cells overexpressed with full-length caspase 3 and μ-calpain cDNAs. Analysis of lysates from caspase 3 and μ-calpain cDNA co-transfected cells revealed a fivefold to sevenfold increase of 120-kd α-fodrin in the level of expression of caspase 3 or μ-calpain in cells transfected with each construct.

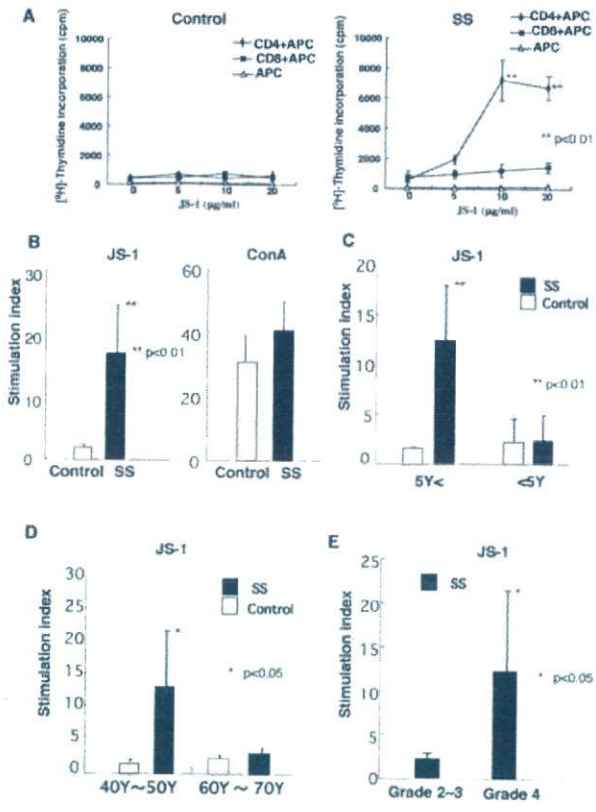

Figure 4. A: Significant proliferative $CD4^+$ T-cell responses, not $CD8^+$ T cells, to the AFN protein (JS-1) in the patients with primary SS ($n = 3$), not in age-matched control ($n = 2$) (**$P < 0.01$, Student's *t*-test). **B:** Significant proliferative responses (stimulation indices > 3) of PBMCs to the AFN protein (JS-1) in patients with primary SS ($n = 14$), not in age-matched control ($n = 11$) (**$P < 0.01$, Student's *t*-test). **C:** Proliferative responses to JS-1 of PBMCs from SS patients with short duration (within 5 years) from the onset of the disease ($n = 8$) were significantly higher than those with long duration (more than 5 years) ($n = 6$) (**$P < 0.01$, Student's *t*-test). **D:** Proliferative responses to JS-1 of PBMCs from younger SS patients (40 to 50 years of age) ($n = 8$) were significantly higher than those with older SS patients (60 to 70 years of age) ($n = 6$) (*$P < 0.05$, Student's *t*-test). **E:** Significant proliferative responses to JS-1 protein were observed in PBMCs from SS patients with higher pathological score ($n = 9$, grade 4) than those with lower score ($n = 5$, grade 2 or grade 3) (*$P < 0.05$, Student's *t*-test). All data are expressed as stimulation indices ± SEM.

PBMCs as antigen-presenting cells. Significant proliferative responses were observed in $CD4^+$ T cells from SS patients, not in $CD8^+$ T cells (Figure 4A). Moreover, it has been determined by flow cytometric analysis that the accumulated population in response to both AFN protein (JS-1) and AFN peptide among PBMCs of SS patients is $CD4^+$ T cell (data not shown). Then, we used PBMCs for the proliferation assay. We found proliferative T-cell responses (stimulation indices > 3) to the AFN protein (JS-1) using PBMCs from 14 of 18 patients with SS, not from age-matched healthy patients ($n = 11$) (Figure 4B). Proliferative responses to JS-1 of SS patients with short duration (within 5 years) from the onset of disease ($n = 8$) were significantly higher than those with long duration (longer than 5 years) ($n = 6$) (Figure 4C). Proliferative responses to JS-1 autoantigen with younger SS patients (40 to 50 years of age) ($n = 8$) were significantly higher than those with older SS patients (60 to 70 years of age) ($n = 6$) (Figure 4D). Significant proliferative responses to JS-1 protein were observed in PBMCs from SS patients with higher pathological score ($n = 9$, grade 4) than those with lower score ($n = 5$, grade 2 or grade 3) (Figure 4E). Synthetic peptides of AFN were generated, and immunoregulatory roles were investigated using PBMCs from patients with SS, compared with SLE and RA. Significant proliferative T-cell responses to AFN peptide were detected in PBMCs from 9 of 18 patients with SS, but not with SLE, RA, and healthy controls (Figure 5). We next analyzed intracellular cytokines using isolated PBMCs (10^6/ml). $CD4^+$ T cells from PBMCs with SS patients induce Th1 cytokine (IL-2, IFN-γ) when pulsed with AFN peptide (10 μg/ml) (Figure 6A), not with control laminin fragment peptide (10 μg/ml). We observed a significant decrease in both $CD4^+$ Fas^+ T and $CD4^+$ $FasL^+$ T cells in SS patients,

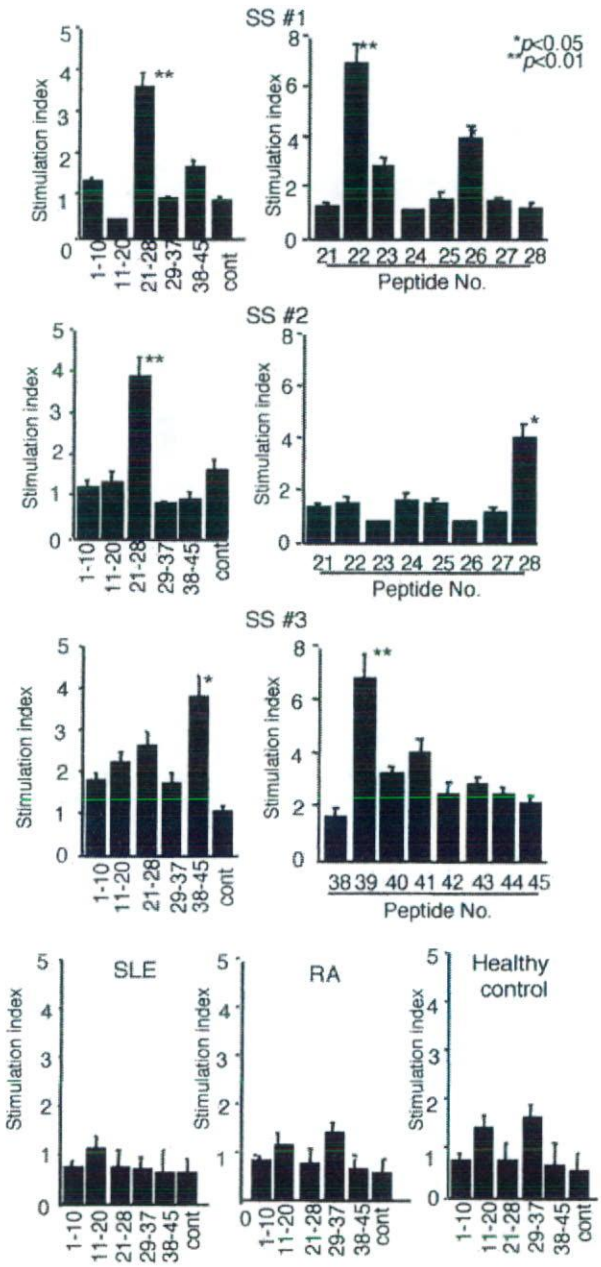

Figure 5. Significant proliferative responses of PBMCs were seen with AFN peptide in the patients with primary SS. Nine of eighteen SS patients examined reacted significantly with single AFN peptide, but not in SLE ($n = 6$), RA ($n = 5$), and age-matched healthy controls ($n = 6$). Representative profiles in three different patients with SS (SS 1, SS 2, SS 3) indicate significant proliferative responses with peptide mixture and individual peptide of p22, p26, p28, and p39 ($^*P < 0.05$, $^{**}P < 0.01$; Student's *t*-test), but not with laminin fragment peptide 929-933 as control antigen. The results are expressed as stimulation indices ± SEM.

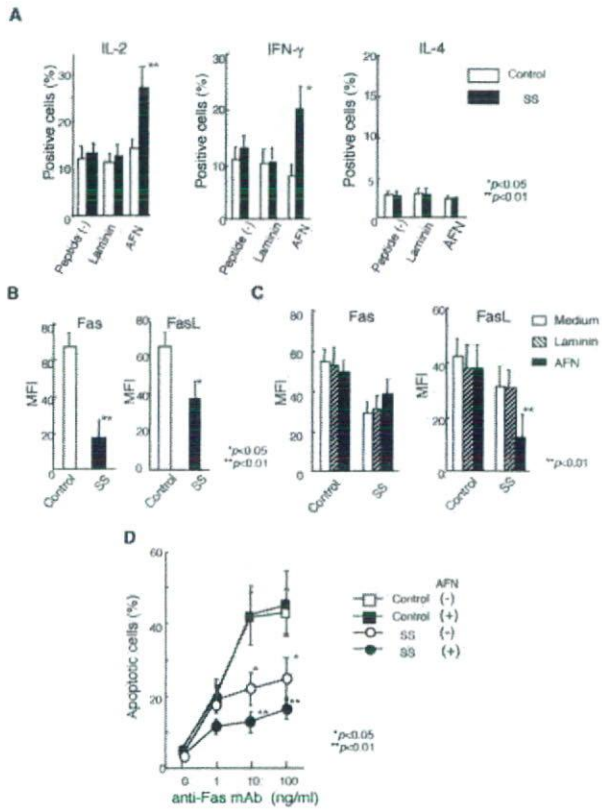

Figure 6. Cytokine profile and Fas-mediated apoptosis in $CD4^+$ T cells from SS patients. **A:** $CD4^+$ T cells from PBMCs with SS patients induce Th1 cytokine (IL-2, IFN-γ), not Th2 cytokine (IL-4) when pulsed with AFN peptide (10 μg/ml) by flow cytometric analysis ($^*P < 0.05$, $^{**}P < 0.01$; Student's *t*-test). Laminin fragment peptide 929-933 (10 μg/ml) was used as control. Five SS patients were analyzed. **B:** Significant decrease in both Fas^+ and $FasL^+$ expression in $CD4^+$ T cells from SS patients, compared with healthy controls ($^*P < 0.05$, $^{**}P < 0.01$; Student's *t*-test). Five SS patients and four healthy controls were analyzed. **C:** AFN peptide-pulsed $CD4^+$ T cells showing significant low intensity of FasL expression, not Fas expression, in SS patients ($^{**}P < 0.01$, Student's *t*-test). MFI (mean fluorescence intensity) indicates the fluorescence intensity of positively stained sample/the fluorescence intensity of its isotype control. Mean fluorescence intensity was calculated using the fluorescence intensity of staining for mAbs to Fas or FasL and isotype-matched control measured by flow cytometry. Five SS patients and four healthy controls were analyzed. **D:** Anti-Fas mAb-stimulated apoptosis showed significant decrease in $CD4^+$ T cells from SS patients than those from healthy control. Moreover, anti-Fas mAb-stimulated apoptosis decreased more significantly in $CD4^+$ T cells from SS patients pulsed with AFN peptide, than those with nonpulsed cells ($^*P < 0.05$, $^{**}P < 0.01$; Student's *t*-test). Five SS patients and four healthy controls were analyzed.

compared with healthy controls (Figure 6B). Moreover, it was demonstrated that AFN peptide-pulsed $CD4^+$ T cells showed a significant low intensity of FasL expression, not Fas expression (Figure 6C). Anti-Fas mAb-stimulated apoptosis showed a significant decrease in $CD4^+$ T cells from SS patients than those from healthy control (Figure 6D). When pulsed with AFN peptide, anti-Fas mAb-stimulated apoptosis decreased more significantly in $CD4^+$ T cells from SS patients.

Discussion

Cleavage of certain autoantigens during apoptosis may reveal immunocryptic epitopes that could potentially induce autoimmune responses in systemic autoimmune diseases.[26,27] We reported previously that a cleavage product of 120-kd α-fodrin may be an important autoantigen on the development of primary SS, and anti-120-kd α-fodrin antibodies have been frequently detected in sera from patients.[9] Although several candidate autoantigens besides α-fodrin have

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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The possible etiopathogenic genes of Sjögren's syndrome

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Abstract

Sjögren's syndrome is a chronic autoimmune disease characterized by focal lymphocytic infiltration of lacrimal and salivary glands, but the precise mechanism of this syndrome is unclear. To clarify the pathogenesis of Sjögren's syndrome, the related genes must be identified. In the present study, we investigate the increased expression of genes and molecules related to Sjögren's syndrome and present our findings of cDNA microarray analysis in the mouse model. Furthermore, we present the results of immunohistochemical analysis of salivary glands in the mouse model and patients with Sjögren's syndrome. This approach might open a new discussion of the existence of principal pathogenic molecules in Sjögren's syndrome.

Keywords: Sjögren's syndrome; MRL/lpr mice; NFS/sld mice; cDNA microarray; Human homologue of SS related genes

Contents

1. Mouse model of Sjögren's syndrome 480
2. cDNA Microarray analysis 480
3. Sjögren's syndrome-related genes and molecules 480
Take-home messages 482
References 483

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1. Mouse model of Sjögren's syndrome

Sjögren's syndrome (SS) is an autoimmune disease characterized by the massive infiltration of lymphocytes into exocrine glands, such as salivary and lacrimal glands, and the subsequent destruction of these exocrine glands. Like other autoimmune diseases, the etiology of SS remains unclear, but previous studies suggest the involvement of hereditary and environmental factors in the onset and progression of the disease. The disease is usually benign and many patients live a typical lifespan. However, the most common symptoms, dry eyes and dry mouth, are problematic and deeply influence patients' quality of life. In addition to these relatively benign manifestations, abnormalities of more vital organs such as renal tubular acidosis, interstitial pulmonary fibrosis, and central nervous system involvement have been demonstrated [1–4]. Therefore, it is important to determine the etiology of SS for the improved management of the disease.

An animal model is one of the most useful tools for studying the pathogenesis of SS; several mouse models have been generated and extensively studied. Among these models, the MRL/lpr mouse bearing the *lpr* gene with a deletion of Fas antigen spontaneously develops systemic vasculitis, glomerulonephritis, arthritis, and sialoadenitis. High levels of autoantibodies, immune complexes, and rheumatoid factor have also been observed in this mouse model [5,6]. Inflammation of the salivary glands in the MRL/lpr mouse is widely accepted as a pathogenic model for human secondary SS [7]. Although the fundamental molecular abnormality in the MRL/lpr mouse model directly depends on the *lpr* gene, the extent of the phenotype and the timing of onset are strongly influenced by background genes [8–10].

The NFS/sld mutant mouse is an animal model of primary SS that bears an autosomal recessive gene that arrests sublingual gland differentiation. Autoimmune sialoadenitis develops when NFS/sld mice undergo a thymectomy 3 days after birth without any immunization (Tx-NFS/sld mice). While no significant inflammatory lesions are observed in other organs or in NFS/sld mice that do not undergo a thymectomy (non-Tx-NFS/sld mice), significant inflammatory changes occur in the salivary glands of Tx-NFS/sld mice 4 weeks after their thymectomy [11,12].

2. cDNA Microarray analysis

Gene expression analysis provides an important perspective on unknown biological phenomena. The following methods are established and applied for basic and clinical studies: differential display [13], suppression subtractive hybridization [14], cDNA microarray hybridization [15], and serial analysis of gene expression (SAGE) [16]. A microarray system is a powerful tool for analyzing the expression profile of thousands of genes in a wide range of biological systems. Recently, microarray analysis has been applied for the research of various clinical disorders such as lymphoma, Huntington's disease, and myocardial infarction, and disease-related genes were isolated in some of these disorders [17–21].

In the present study, we isolated genes that contribute to the progression of SS, using mRNA from SS model mouse salivary glands and an in-house cDNA microarray, and identified up-regulated genes.

3. Sjögren's syndrome-related genes and molecules

To investigate the gene expression profile in SS, we examined the mRNAs of the MRL/lpr and NFS/sld mouse salivary glands using cDNA microarrays. We arrayed a set of 4608 cDNA clones derived from oligo-capped mouse brain, fetus, kidney, and spleen. The most aggressive inflammation in the salivary gland of MRL/lpr mouse occurs at the age of 12–16 weeks [8,22], so we compared the mRNAs of MRL/lpr and MRL/++ mouse salivary glands at the age of 16 weeks. We identified 15 highly expressed genes [*IL-16*, *Grap*, *caspase3*, *Ly-6C.2*, *Mel-14 antigen*, *cathepsin B*, *mpt1*, *Laptm5*, *Gnai2*, *vimentin*, *UCP2*, *saposin*, *Trt*, *laminin receptor 1*, *and HSP 70 cognate*] in the salivary gland of MRL/lpr mouse by cDNA microarray analysis, which were likely to be SS-related genes [23] (Table 1).

We performed reverse transcription-polymerase chain reaction amplification to confirm the high expression of the following 15 genes. High expression was verified in 11 of the 15 genes: *IL-16*, *Grap*, *caspase3*, *Ly-6C.2*, *vimentin*, *Mel-14 antigen*, *cathepsin B*, *mpt1*, *Laptm5*, *Gnai2*, and *UCP2*. Five of these genes (*caspase 3*, *Ly-6C*, *vimentin*, *Mel-14 antigen*, and *cathepsin B*) have already been recognized in patients with SS or the SS mouse model [24–29].

Table 1
Highly expressed genes in MRL/lpr mice salivary gland in comparison with MRL/++

Accession No.	Name of genes	Fold change[a]
NM_009810	Mus musculus *caspase 3*	2.31
M18466	Mouse lymphocyte differentiation antigen *Ly-6C.2*	2.75
M26251	Mouse *vimentin*	2.21
M25324	Mouse peripheral lymph node-specific homing receptor (*MEL-14* antigen)	3.50
NM_007798	Mus musculus *cathepsin B*	1.84
AF006467	Mus musculus membrane-associated phosphatidylinositol transfer protein (*mpt1*)	1.98
NM_010686	Mus musculus lysosomal-associated protein transmembrane 5 (*Laptm5*)	2.16
NM_008138	Mus musculus guanine nucleotide binding protein, alpha inhibiting 2 (*Gnai2*)	1.93
U69135	Mus musculus *UCP2*	2.06
S36200	Mouse saposin=sphingolipid activator protein	1.88
NM_009429	Mus musculus translationally regurated transcript (*Trt*)	1.82
NM_011029	Mus musculus laminin receptor 1 (*Lamr1*)	2.02
M19141	Mouse heat shock protein 70 cognate	1.61
AF175292	Mus musculus neuronal *IL-16*	2.20
NM_027817	GRB2-related adaptor protein (*Grap*), mRNA	1.85

[a] The averages of the fold change based on the normalized microarray fluorescent data of MRL/lpr compared to MRL/++ ($n=8$).

Although a high expression of *caspase 3* has been reported in the NOD mouse model of SS [24], the MRL/lpr mouse is Fas-deficient and thus lacks Fas/Fas ligand pathway-dependent apoptosis. This suggests that Fas/Fas ligand pathway-independent apoptosis, such as perforin-or granzyme-dependent apoptosis [30], is induced in MRL/lpr mouse salivary glands. One of the adaptor molecules, Grap, effectively delivers signals from the immune cell surface to a downstream functional molecule. Grap has a structural arrangement of an SH3-SH2-SH3 domain, which is similar to that of other immune cell adaptor molecules such as Grb2, Gads, and Grap2 [31]. Grap is known to be specifically expressed in lymphoid tissues, and structurally resembles Grb2 more than other Grb2 family molecules in that Grap does not have the proline-rich motif. By immune cell activation, Grap binds to phosphorylated tyrosine of the local area transport (LAT) at its SH2 region, and further binds to Son of sevenless (Sos) in a manner similar to that of Grb2. Further down-stream events remain unknown. We have observed that the expression of Grap in the salivary glands of the model mice was higher than that of the control mice. Furthermore, we have identified 7 genes in the spleen of MRL/lpr mice not found in the spleen of MRL/+ mice using the mouse spleen cDNA microarray chip [32] (Table 2). Namely, the *Grap* gene was commonly up-regulated in the spleen and salivary glands from MRL/lpr mice [32]. Immunohistochemical staining in the salivary gland revealed substantial differences between MRL/lpr and MRL/++ in the expression of mouse Grap. Furthermore, the immunohistochemical staining of specimens from 3 patients with SS and 2 controls (subjects with salivary cysts) indicated that the human homologue of Grap was expressed on ductal cells and on certain infiltrating cells in patients with SS, but very weakly in the controls [32]. These results may suggest that in diseased salivary glands and spleen, enhanced stimulation of T cell receptor augments signal transduction to downstream molecules associated with apoptosis. Further detailed analysis of the Grb2 family may clarify the regulation of T cell differentiation and apoptosis in SS.

Table 2
Highly expressed genes in MRL/lpr mice spleen in comparison with MRL/++

Accession No.	Name of genes	Fold change[a]
U88682	Mouse anti-DNA antibody heavy chain variable region mRNA	2.88
XM_134565	Mouse similar to Gag-Pol polyprotein mRNA	1.93
M16072	Mouse Ig active gamma-2a H-chain V-Dsp2.2-J2-C mRNA	1.64
BC036286	Mouse myeloid/lymphoid or mixed-lineage leukemia 5, mRNA	2.17
NM_025408	Mouse phytoceramidase, alkaline (Phca)mRNA	3.23
X76772	Mouse mRNA for ribosomal protein S3	1.56
NM_027817	Mus musculus GRB2-related adaptor protein (*Grap*), mRNA	2.82

[a] The averages of the fold change based on the normalized microarray fluorescent data of MRL/lpr compared with MRL/+ ($n=6$).

To our knowledge, the remaining five genes (*mpt1, Laptm5, UCP2 Gnai2* and *IL16*) have not been identified previously as SS-related genes. *Mpt1* was cloned as a mouse homologue of Drosophila retinal degeneration B (rdgB), and the mpt1 protein has been predicted to be a membrane-bound phosphatidylinositol transfer protein (PITP) [33], which transports phosphatidylinositol (PI) through the aqueous phase from one membrane compartment to another and functions as a cofactor for the synthesis of phosphatidylinositol bisphosphate (PIP2) [34]. Given that the constitutive turnover of PI is markedly augmented in some subsets of T lymphocytes in MRL/lpr mice [35], it is fair to speculate that such T cells accumulate and are related to the pathogenesis of SS. *Laptm5* is highly expressed in adult hematopoietic organs such as bone marrow, spleen, thymus, lymph nodes, and peripheral blood leukocytes [36]. The high expression of *Laptm5* in our MRL/lpr mouse model of SS could be due to an increased number of lymphocytes infiltrating the salivary glands. Further experimental studies are required to clarify the role of *Laptm5* in the pathogenesis of SS. The mitochondrial protein known as uncoupling protein 2 (*UCP2*) is highly expressed in the spleen and macrophages. A recent report suggests that *UCP2* plays a role in limiting macrophage-mediated immunity [37]. The expression of *UCP2* is induced by TNF-α [38] and the expression of TNF-α is increased in MRL/lpr mice [39]. These combined findings suggest that increased TNF-α in MRL/lpr mouse salivary glands could contribute to the up-regulation of *UCP2* and subsequent disease progression.

We then examined the expression of these genes in the salivary glands of MRL/lpr mice and NFS/sld mice that had undergone a thymectomy (Tx-NFS/sld), a new model for primary SS, by using real-time-quantitative reverse transcription-polymerase chain reaction analysis. The expression of 11 genes (*IL-16*, *Grap*, *caspase3*, *Ly-6C.2*, *Mel-14 antigen*, *cathepsin B*, *mpt1*, *Laptm5*, *Gnai2, vimentin,* and *UCP2)* was higher in MRL/lpr mice than in MRL/++ mice and the expression of 9 genes (*IL-16*, *Grap*, *caspase3*, *Ly6c2, Mel-14 antigen*, *cathepsinB*, *mpt1 Laptm5,* and *Gnai2)* was higher in the Tx-NFS/sld mice than in the control mice that did not undergo thymectomy. In addition, the fetus microarray analysis demonstrated that the *Laptm5* gene was also highly expressed in the salivary glands of Tx-NFS/sld mice. Furthermore, immunohistochemical studies showed that mouse and human *Laptm5* protein antigens were expressed on certain infiltrated lymphocytes and on ductal cells in the salivary glands from the SS mouse model and patients with SS, but very weakly in control subjects. These results suggest that some apoptosis-related genes might be responsible for the pathogenesis of organ-specific autoimmune lesions in SS (Arthritis Rheumatism Vol. 9, S251, 2001, Arthritis and Rheumatism Vol. 50 S577 2004).

These early findings confirm the excellent specificity and reproducibility of our cDNA microarray analysis for identification of disease-related genes. A microarray system can handle thousands of genes and help in the extraction of genes that have significant relationships to the stages of a disease. In addition, an in-house cDNA microarray is advantageous in allowing the exchange of arrayed genes and assaying specific disease-related genes on one glass slide, which is useful for the diagnosis and prediction of clinical stages.

In the present study, we isolated nine SS-related genes on a cDNA microarray using the MRL/lpr SS mouse model. Further studies may allow the identification of other SS-related genes, thus allowing the performance of clustering analysis, which could provide useful information about classification of the disease, clinical course, stage of the disease, and selection of a suitable treatment. The combination of an in-house microarray and the use of an animal model is a suitable strategy for exploring a gene expression profile and should gradually evolve into a system useful for clinical investigation.

Take-home message

- The combination of cDNA microarray and use of animal models is a suitable strategy for exploring a gene expression profile and should gradually evolve into a system useful for clinical investigation.
- Fifteen highly expressed genes, *IL-16*, *Grap*, *caspase3*, *Ly-6C.2*, *Mel-14 antigen*, *cathepsin B*, *mpt1*, *Laptm5*, *Gnai2, vimentin*, *UCP2*, *saposin*, *Trt*, *laminin receptor 1*, and *HSP 70 cognate*, in the salivary gland of MRL/lpr mouse by cDNA microarray analysis were identified.
- The expression of 9 genes, *IL-16*, *Grap*, *caspase3*, *Ly6c2, Mel-14 antigen*, *cathepsin B*, *mpt1 Laptm5*, and *Gnai2,* was higher in the salivary gland of