presentation of antigen on the cell surface begin with synthesis of class II $\alpha\beta$ heterodimers in the endoplasmic reticulum. These class II $\alpha\beta$ heterodimers associate early during biosynthesis with a type II membrane protein, the invariant chain (Ii) (Roche and Marks, 1991; Lamb and Cresswell, 1992). Several lysosomal proteases have been implicated in the processing of Ii and antigenic peptides. Cathepsin B, the most abundant lysosomal cysteine protease, has been tied to Ii degradation using purified class II-Ii complexes (Reyes et al., 1991). Cathepsin L, a potent cysteine-class endoprotease, is specifically inhibited by a fragment of the alternatively spliced Ii form p41 (Bevec et al., 1996). Cathepsin S containing potent endoproteolytic activity is highly expressed in the spleen and professional APCs and other class II-positive cells, and is inducible with by IFN-γ (Shi et al., 1992, 1994). Katunuma and his colleagues have developed specific inhibitors of cathepsin B (CA074), cathepsin L (Clik148), and cathepsin S (Clik60), in vivo as well as in vitro (Towatari et al., 1991; Murata et al., 1991; Katunumua et al., 1999). Matsunaga et al. first reported that CA074 suppresses immune responses (Matsunaga et al., 1993), suggesting that cysteine proteases in lysosomes play an important role in the functional differentiation of MHC class II-restricted $CD4^+$ T cells. However, it is uncertain whether the inhibition of cathepsins block generation of the antigenic peptide on the development of autoimmune diseases. To address this important issue, autoantigen processing and presentation after specific inhibition of cathepsins were examined in murine model for SS and IDDM.

Materials and methods

Mice

Female NFS/N strain carrying the mutant gene *sld* (Hayashi et al., 1988), and NOD mice were reared in our specific pathogen-free mouse colony and given food and water ad libitum. Thymectomy was performed on day 3 after birth (3d-Tx) in NFS/*sld* mice. C56BL/6 mice (Charles River Japan, Inc., Atsugi, Japan), were used as controls.

Cyclophosphamide-induced diabetes

Prediabetic (7–8-week-old) female NOD mice were challenged with i.p. injection of cyclophosphamide (CY) (200 mg/kg) (Sigma-Aldrich) and analyzed 1–4 weeks after CY injection.

Assessment of diabetes

The blood glucose level was monitored weekly with a Glucometer (Kodama, Tokyo, Japan) using 50 μl blood from tail vein. Mice were monitored weekly for development of urine glucose with Keto-Diastix (Bayer-Sankyo Co., Ltd., Tokyo, Japan).

Cathepsin inhibitors

Specific inhibitors for cathepsin B (CA074), cathepsin L (Clik148), and cathepsin S (Clik60) have been developed with the help of computer-graphic modeling based on the stereo-structure as described previously (Towatari et al., 1991; Murata et al., 1991; Katunumua et al., 1999).

In vivo treatment with cathepsin inhibitors

We examined the in vivo therapeutic effects of cathepsin S-inhibitor (Clik60), cathepsin B-inhibitor (CA074), and cathepsin L-inhibitor (Clik148) in murine model for SS and IDDM in CY-treated NOD mice. Each inhibitor (Clik60, CA074, and Clik148) dissolved in PBS was administered i.p. into model mice at doses of 0.1 mg/mouse/day) and compared with non-treated model mice. All organs were removed from the mice, fixed with 4% phosphate-buffered formaldehyde (pH 7.2) and prepared for histological examination. The sections were stained with hematoxylin and eosin (H&E). Histological grading of the inflammatory lesions in the SS model was done according to the method proposed previously (White and Caserett, 1974).

Proliferation assay

Single cell suspensions of spleen cells or regional lymph node cells (LNCs) were cultured in 96-well flat bottom microtiter plates (Nunc, Roskilde, Denmark). For proliferation assay, a total of 5×10^5 cells/well were cultured for 72 h under stimulation of recombinant α-fodrin protein (JS-1, 10 μg/ml) (10), insulin (10 μg/ml), GAD (10 μg/ml), OVA (10 μg/ml), and ConA (5 μg/ml), and pulsed with 1 μCi/well of [^{3}H]thymidine (NEN Life Science Products, Boston, MA) during final 20 h of the culture. We further examined the in vitro preventive effects of cathepsin inhibitors (10^{-7}–10^{-4} M CA074, Clik148, and Clik60) for antigen-specific proliferative T cell responses. T cell purification was done using CD4mAb-bounded immunomagnetic beads (Dynal, CA).

Measurement of cytokine production

Cytokine production from spleen cells was tested by two-step sandwich ELISA using a mouse IL-2, IL-4, and IFN-γ kit (Genzyme). In brief, culture supernatants from spleen cells activated with immobilized anti-CD3 mAb (10 μg/ml)(Ceder Lane Lab.) for 3 days 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. Plates were washed extensively with 1% Tween in PBS between each step. Finally, 2,2′-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) substrate containing H_2O_2 was added and the colorimetric reaction was read at an absorbance of 450 nm using an automatic microplate reader (BioRad Laboratories Inc., Hercules, CA). The concentrations of IL-2 (pg/ml), IL-4

(pg/ml), and IFN-γ pg/ml) were calculated according to the standard curves produced by various concentrations of recombinant cytokines.

Detection of serum autoantibodies against 120 kD α-fodrin

Serum autoantibody production against 120 kD α-fodrin was analyzed by immunoblotting as described previously (Haneji et al., 1997).

Measurement of fluid secretion

Detection of tear and saliva volume of the treated, and non-treated SS animal model was done according to a modified method as described (Delporte et al., 1997).

Results and discussion

Therapeutic effects of specific inhibitors of Cathepsin S (Clik60) for animal model of SS

To address the role of autoantigen-reactive T cells, we examined the proliferative T cell responses in the cervical LNCs and spleen cells from SS model mice and controls. We found that the LNCs and spleen cells in SS model mice at 8 weeks of age showed a significant increase in autoantigen (JS-1)-specific T cell proliferation (Fig. 1a), but not in C57BL/6 control mice. No significant differences were observed in the proliferative response stimulated with OVA (10 μg/ml), and ConA (5 μg/ml) among these mice. We examined the inhibitory effects of cathepsin inhibitors against autoantigen-specific T cell responses in vitro. In regional LNCs, but not in the spleens, from SS model mice, a significantly inhibitory effect of Clik60 was observed in dose-dependent manner (Fig. 1b). We next examined the in vivo therapeutic effects of Clik60, Clik148, and CA074 in murine model for SS. Treatment with i.p. injection of Clik60 (0.1 mg/mouse/day) was effective in preventing the development of autoimmune lesions in the lacrimal ($P<0.01$), parotid ($P<0.05$), and submandibular ($P<0.05$) glands of the SS model mice, but not in groups injected with CA074, and Clik148 (Fig. 2a). Moreover, the average saliva and tear volume of Clik60-treated model mice was significantly higher than that of the non-treated SS model mice (Fig. 2b). In addition, autoantigen (JS-1)-specific T cell response was significantly inhibited in LNCs from Clik60-treated mice. Culture supernatants from anti-CD3mAb-stimulated splenic T cells obtained from Clik60-treated mice contained high levels of IL-4, but low levels of IL-2 and IFN-γ by ELISA (Fig. 3a). Serum autoantibody production against 120 kD α-fodrin autoantigen was exclusively inhibited in Clik60-treated mice, but not in other groups (Fig. 3b). These results strongly suggest that Clik60 plays an important role in preventing autoantigen presentation to generate class II molecules competent for binding antigenic peptide, resulting in inhibition of autoimmunity in the salivary and lacrimal glands. A large proportion of class II ($I\text{-}A^q$)-expressing cells was observed

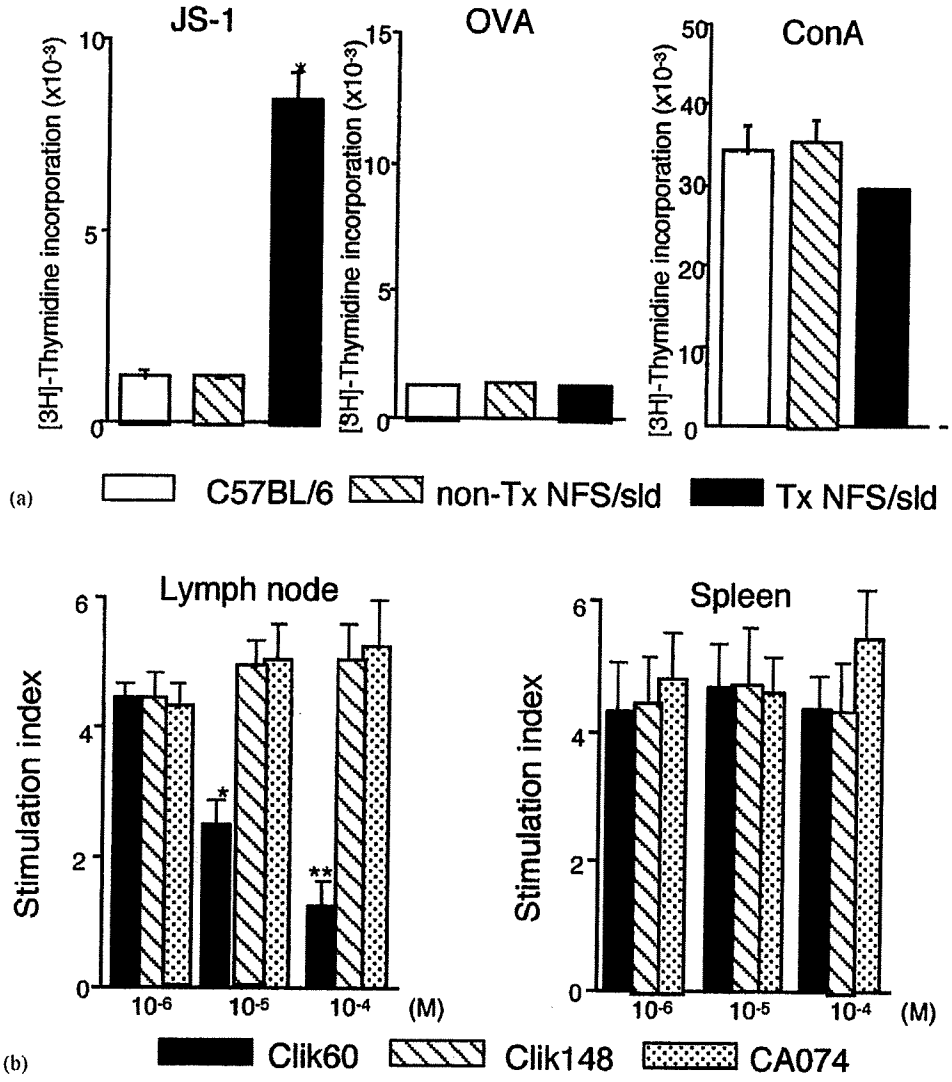
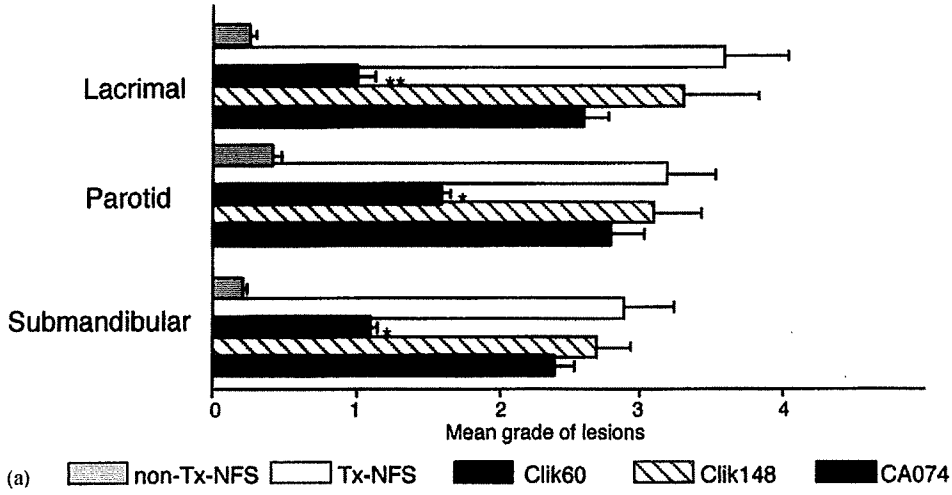


Fig. 1. (a) Detection of proliferative T cell response of LNCs from SS model mice to organ-specific autoantigen (JS-1) (*$P<0.001$, Student's t-test)(a). No differences were found in OVA (10 μg/ml), and ConA (5 μg/ml)-responsiveness. (b) In vitro preventive effect of proliferative T cell response of LNCs, but not spleen cells, to JS-1 by Clik60 in dose-dependent manner. No inhibitory effects of Clik148, and CA074 were found. Data are expressed as stimulation indices (SI)±standard error of the mean (s.e.m.). Three experiments from each group were performed at 8 weeks of age, and the mean values of index were statistically significant at $P<0.05$* and $P<0.01$** (Student's t-test). Part of this Figure included in reference by Saegusa et al. (2002).



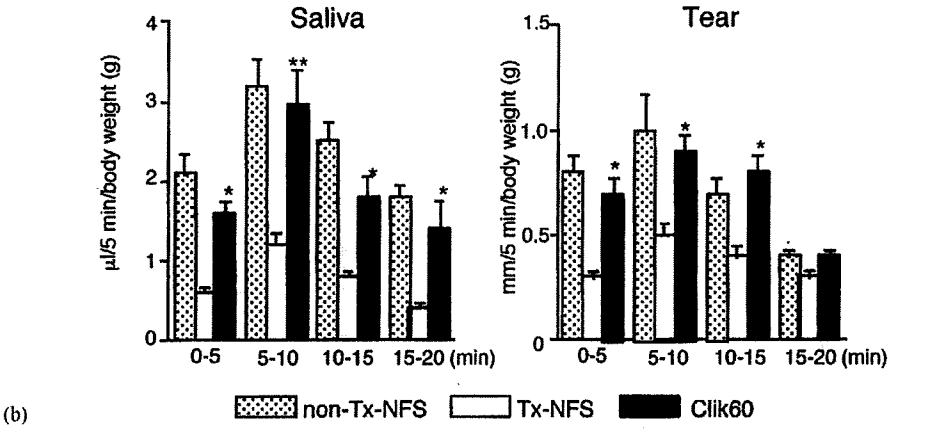


Fig. 2. (a) Effects of in vivo administration of i.p. injection of cathepsin inhibitors (Clik60, Clik148, and CA074). Each inhibitor was administered into SS model mice ($n = 10$, for each) at doses of 0.1 mg/mouse/day from 4 weeks–7 weeks, and then analyzed at 8 weeks, compared with non-treated SS model mice ($n = 7$). The treatment with i.p. injection of Clik60 was effective in preventing the development of autoimmune lesions in the lacrimal, parotid, and submandibular glands of the SS model mice, but not in groups injected with Clik148, and CA074 ($^{*}P<0.01$ and $^{**}P<0.005$, Student's t-test). (b) The average saliva and tear volume of the SS model mice treated with Clik60 was significantly higher than that of control mice. Results are expressed as mean±SEM in 5 mice examined per each group ($^{*}P<0.05$ and $^{**}P<0.005$, Student's t-test). Part of this Figure included in reference by Saegusa et al. (2002).

on mouse salivary gland (MSG) epithelial cells from SS model mice, and MHC class II molecule can be stably induced by IFN-γ-stimulation on MSG cells from syngeneic control mice (Saegusa et al., 2002). It is possible that the salivary gland

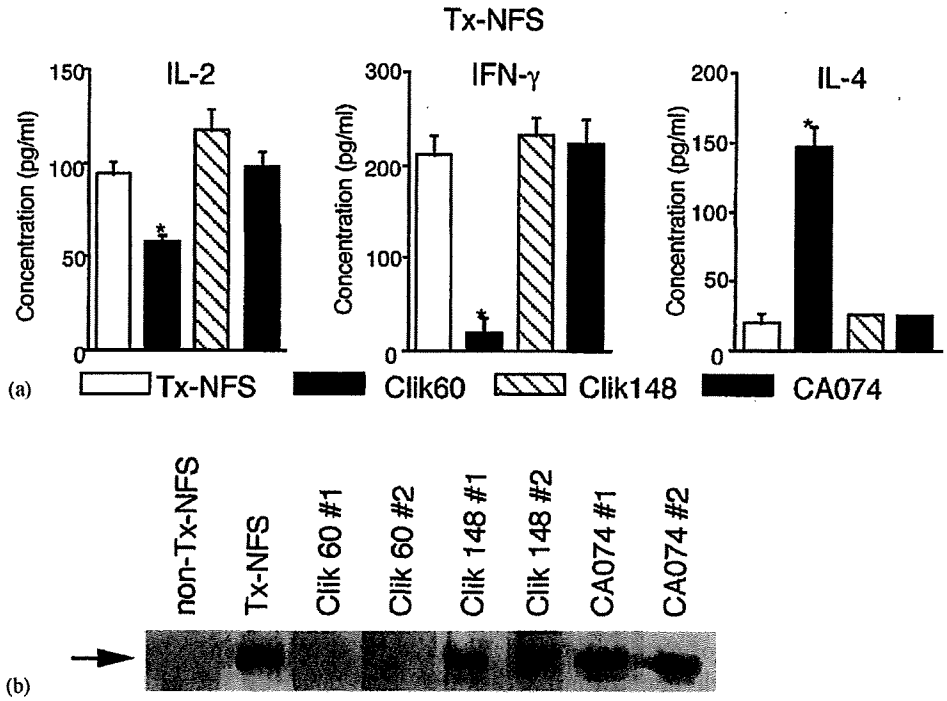


Fig. 3. (a) Down-regulation of the Th1-type cytokine production in the SS model mice treated with Clik60. Culture supernatants from anti-CD3 mAb-stimulated splenic T cells from Clik60-treated model mice (8-week-old) contained a high level of IL-4, but low levels of IL-2 and IFN-γ as measured by ELISA ($^{*}P<0.005$ and $^{**}P<0.001$, Student's t-test). (b) Inhibition of serum autoantibody production to 120 kD α-fodrin was observed in two different recipient mice treated with Clik60 (#1, #2) as shown by immunoblotting. Part of this Figure included in reference by Saegusa et al. (2002).

epithelial cells may function, at least in part, as autoantigen-presenting cells on the development of murine SS, and that inhibition of cathepsin S prevents the autoantigen presentation and subsequent peptide binding by class II molecules. MHC class II molecules bind a diverse array of peptides derived from the endocytic pathway and present them to $CD4^{+}$ T cells. MHC class II molecules are synthesized with their peptide-binding site blocked by Ii, and they acquire the capacity to bind antigens only after Ii has been degraded in the compartments. The treatment with cathepsin S-inhibitor was effective in preventing the development of autoimmune lesions in the SS model mice. In addition, Clik60-treated mice showed a significant downregulation of autoantigen (JS-1)-specific T cell response and Th1 cytokine expressions. These results indicate that cathepsin S-inhibitor plays an important role for preventing autoantigen presentation that followed by inhibition of autoimme exocrinopathy in SS.

Therapeutic effects of specific inhibitors of Cathepsin L (Clik148) for Autoimmune Diabetes (NOD) mice

We next examined the in vivo therapeutic effects of Clik60, Clik148, and CA074 in NOD mice, a well-known strain of animal model for autoimmune diabetes. Treatment with i.p. injection of Clik148 (0.1 mg/mouse/day) was effective in preventing the glucose production in blood and urine from the cychrophosphamide (CY)-treated NOD mice, but not in groups injected with CA074, and Clik60 (0.1 mg/mouse/day) (Fig. 4). To ensure the role of autoantigen-reactive T cells, we examined the proliferative T cell responses in the peripancreatic LNCs and spleen cells from CY-NOD mice and C57BL/6 control mice. We found that the peripancreatic LNCs and spleen cells in CY-NOD mice showed a significant increase in autoantigen (GAD and insulin)-specific T cell proliferation, but not in control mice. No significant differences were observed in the proliferative response stimulated with OVA (10 μg/ml) and ConA (5 μg/ml) among these mice. Then, we examined the inhibitory effects of cathepsin inhibitors against autoantigen-specific T cell responses in vitro. In peripancreatic LNCs from CY-NOD mice, a significantly inhibitory effect of Clik148 was observed in both anti-GAD and anti-insulin T cell responses, but no effects were found in incubation with Clik60 or CA074 (Fig. 5). Moreover, surface expressions of class II molecule induced by IFN-γ, and TNF-α stimulation in mouse islet β-cell line (Min6) was clearly inhibited by the incubation with Clik148, but not with Clik60 and CA074 determined by flow cytometry (Fig. 6). From these results, it was strongly suggested that Clik148 play a significant role in preventing autoantigen presentation to generate class II molecules competent for binding antigenic peptide, resulting in inhibition of autoimmune diabetes in NOD mice.

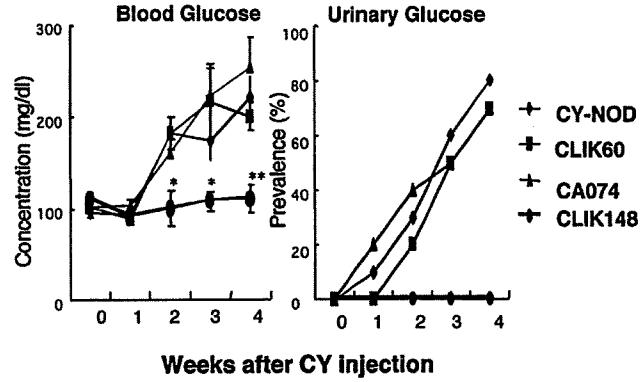


Fig. 4. Treatment with i.p. injection of Clik148 (0.1 mg/mouse/day) was effective in preventing the secretion of blood glucose and urine glucose from the cychrophosphamide (CY)-treated NOD mice ($^{*}P<0.05$, $^{**}P<0.01$, Student's *t*-test). The blood glucose level was monitored weekly with a glucometer using 50 μl blood from tail vein, and urine glucose was monitored with Keto-Diastix.

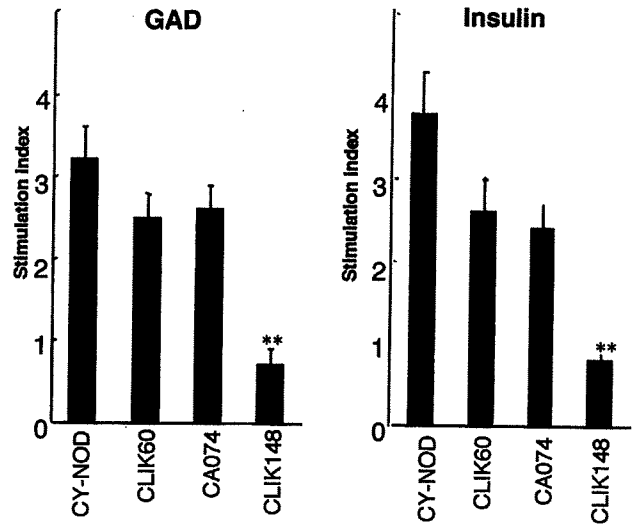


Fig. 5. Autoantigen (GAD and insulin)-stimulated proliferative T cell response of peripancreatic LNCs from NOD mice was significantly inhibited by incubation with Clik148, but not with Clik60, and CA074 (**$P<0.01$, Student's t-test). Data are expressed as stimulation indices (SI) ± standard error of the mean (s.e.m.). Three experiments from each group were performed.

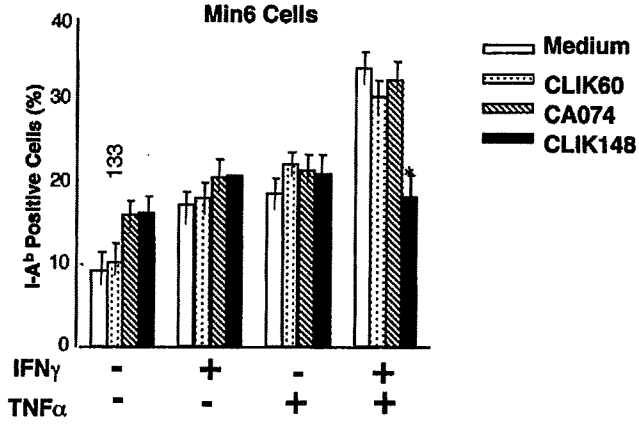


Fig. 6. Expression of MHC class II molecule induced by IFN-γ (100 U/ml), and TNF-α (100 U/ml)-stimulation in mouse islet β-cell line (Min6) was significantly inhibited by the treatment with Clik148, but not with Clik60, and CA074 (**$P<0.01$, Student's t-test). Three experiments from each group were performed.

Differential effect of cathepsin S and L on Antigen presentation

Recent studies have shown that cathepsin S and cathepsin L are important for Ag presentation by discrete populations of cells (Nakagawa et al., 1998, 1999; Shi et al.,

1999). Active cathepsin S was detected in B cells, dendritic cells (DCs), and peritoneal macrophages, where it was shown to be involved in the late stages of Ii degradation (Shi et al., 1999). By contrast, cathepsin L was detected only in macrophages and cortical thymic epithelial cells, where a defect resulted in severely impaired $CD4^+$ T cell selection (Nakagawa et al., 1998). Thus, it would appear that different APCs utilize distinct cathepsins to mediate late stage Ii degradation and regulate MHC class II presentation. Moreover, cathepsin S-deficient mice showed diminished susceptibility to collagen-induced arthritis, suggesting a potential therapeutic target for regulation of immune responsiveness (Nakagawa et al., 1999). Because of this cell type-restricted expression, cathepsin L deficiency results in diminished positive selection of $CD4^+$ T cells but does not significantly affect Ag presentation by bone marrow-derived APCs (Nakagawa et al., 1998). In contrast to Ii degradation, little is known about Ag processing, i.e., the proteolytic mechanisms that generate particular T cell epitopes. Early studies found that inhibition of lysosomal acidification interferes with proteolysis and Ag presentation, implicating lysosomal proteases (Watts, 1997). Evaluation of cathepsin L and cathepsin S has focused on their roles in Ii degradation and not in Ag processing (Villadangos and Ploegh, 2000). Recent evidence has demonstrated that several cathepsins are expressed in a tissue-specific fashion and that partial proteolysis of specific biological targets is a key function of cathepsins in antigen processing. We confirmed that mouse salivary gland cells and pancreas β islet cells might play an important role for presenting autopeptide in the autoimmune responses, and that inhibition of cathepsin S and cathepsin L prevents the surface expression of peptide/class II complex formation. The differential expression of proteinases by distinct APCs may affect the types of autoantigen peptides that are presented to T cells and thereby the immune responses that are ultimately generated. Our experiments support the hypothesis that cathepsin S, and cathepsin L, previously shown to be important in Ii processing in vitro, regulates MHC class II function and subsequent autoimmune responses in vivo. Thus, selective inhibition of cysteine proteases cathepsin S and cathepsin L may have important therapeutic potential in modulating class II—restricted autoimmune processes.

Summary

The cysteinal lysosomal proteases, cathepsin S and cathepsin L have been shown to process invariant chain, thereby facilitating MHC class II maturation. However, their role in antigen processing is not established. Studies examined the functional significance of cathepsin inhibition on antigen processing and autoimmune diseases in murine models for SS and non-obese type-I diabetes model (NOD). Specific inhibitor of cathepsin S (Clik60) in vitro markedly impaired presentation of an organ-specific autoantigen, 120 kD α-fodrin, by interfering with MHC class II–peptide binding. Antigen-specific T cell responses were significantly inhibited by incubation with Clik60 in dose-dependent manner. Ttreatment with Clik60 in vivo profoundly blocked lymphocytic infiltrations in the salivary and lacrimal glands and

abrogated a rise in serum autoantibody production. Moreover, treatment with i.p. injection of specific inhibitor of cathepsin L (Clik148) was effective in preventing the glucose production in blood and urine from NOD mice, but not in groups injected with CA074 and Clik60. Clik148 markedly impaired presentation of autoantigens including insulin and GAD in NOD mice. Thus, inhibition of cathepsin activity in vivo alters autoantigen presentation and development of autoimmunity. Our experiments demonstrate that selective inhibition of cysteine proteases is an additional potential strategy for modulating the autoantigen-derived immune response in class II—restricted autoimmune diseases including Sjögren's syndrome and autoimmune diabetes.

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

Review Article

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

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

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

Introduction

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

Received 9/29/04; accepted 10/22/04.
Grant support: Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan
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not known whether disease is initiated by a restrained inflammatory reaction to an organ-specific autoantigen. In most cases, antigenic challenge results in the establishment of immunological memory, a state in which the immune system is maintained to respond effectively upon recurrent antigenic exposure. It is now evident that the interaction of Fas with FasL regulates a large number of pathophysiological processes of apoptosis including autoimmune diseases[7,8]. Recent studies have now confirmed the observation that apoptotic cells in various cell types are implicated as the source of autoantigen when stimulated with different proapoptotic stimuli[9-11]. Apoptosis results in surface accessibility of all SSA/Ro-SSB/La antigens for recognition by circulating maternal antibodies. Although cleavage of certain autoantigens during apoptosis may reveal immunocryptic epitopes that could potentially induce autoimmune responses in systemic autoimmune diseases[12,13], accumulated evidence suggests an important role of apoptosis in the disease pathogenesis of Sjögren's syndrome[14].

Apoptotic cells in target organs

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

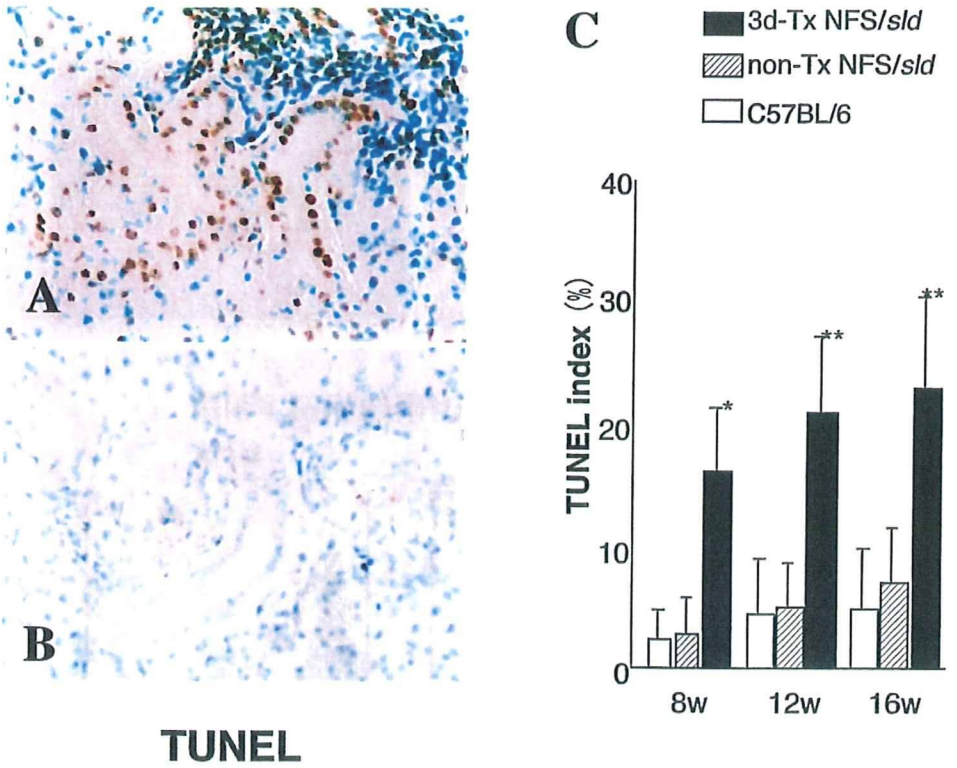

Fig. 1 Tissue-specific apoptosis in the salivary glands in Sjögren's syndrome mouse model[31]. (A) In situ TUNEL detection was frequently positive for epithelial duct cells in 3d-Tx NFS/*sld* mice (12-wk-old) (A), but not in non-Tx NFS/*sld* mice (12-wk-old) (B). (C) A significant increase of apoptotic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

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

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

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

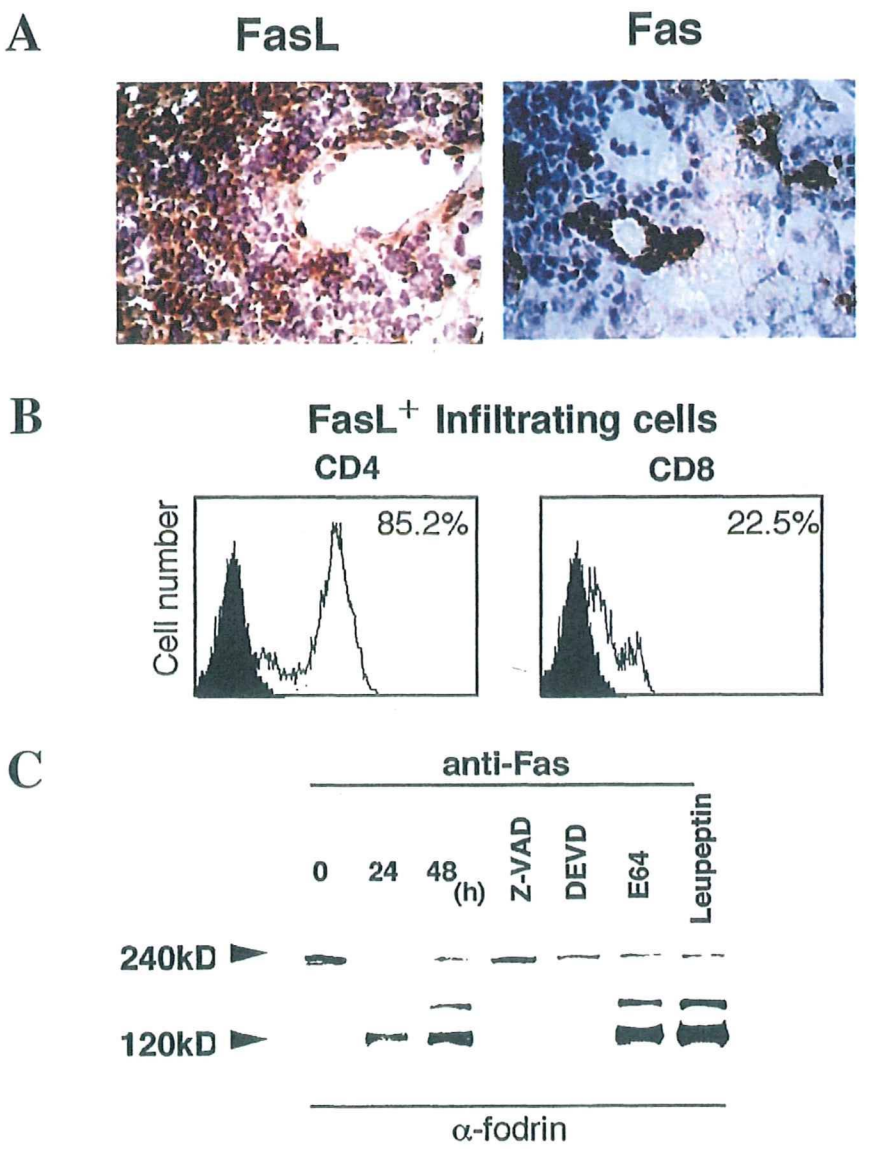

Fig. 2 FasL and Fas expression on tissue-infiltrating cells in the salivary gland tissues from 3d-Tx NFS/*sld* mice[31]. (A) Immunohistochemical detection of infiltrating lymphocytes strongly positive for FasL in 3d-Tx NFS/*sld* mice. Epithelial duct cells were stained positively with Fas in 3d-Tx NFS/*sld*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

of Sjögren's syndrome, but not from Fas-deficient MRL/lpr mice and FasL-deficient MRL/gld mice. FasL undergoes metalloproteinase (MMP)-mediated proteolytic processing in its extracellular domains, resulting in the release of soluble trimeric ligands (soluble FasL, sFasL). In this case, the processing of sFasL occurs in autoantigen-specific $CD4^+$ T cells, and a significant increase in expressions of MMP-9 mRNA was observed in spleen cells from the mouse model of Sjögren's syndrome[46]. *In vitro* T cell apoptosis assay indicated that FasL-mediated AICD is down-regulated by autoantigen stimulation in the murine SS model. The data indicate that the increased generation of soluble FasL inhibits the normal AICD process, leading to the proliferation of effector $CD4^+$ T cells[46]. Our study demonstrates that an organ-specific autoantigen may play an important role in the down-modulation of FasL-mediated AICD. The increased generation of sFasL inhibits the normal AICD process, leading to the proliferation of effector $CD4^+$ T cells in the murine SS model. Previous studies have demonstrated that $CD4^+$ T cells are susceptible to AICD induced through TCR-mediated recognition of allogeneic MHC class II molecules, supporting the notion that AICD can be triggered in activated T cells through the TCR-mediated recognition of antigen[47-49]. Mice or human individuals lacking functional Fas or FasL display profound lymphoproliferative reactions associated with autoimmune disorders[7,50]. In proteoglycan-induced arthritis, $CD4^+$ T cells proliferate at a high rate in response to proteoglycan stimulation, and exhibit a Th1-type response[51]. The study investigated whether when Th1 cells were dominant, disease outcome could be modified with pharmacological amounts of Th2 cytokines. Treatment with IL-4 prevented disease and induced a switch from a Th1-type to a Th2-type response. Proinflammatory cytokine mRNA transcripts were reduced in joints of cytokine-treated mice. Th2 cytokine therapy at the time of maximum joint inflammation also suppressed symptoms of disease. These observations have suggested that a defect in AICD of autoreactive Th1 cells may contribute to the pathogenesis of Sjögren's syndrome. $CD4^+$ T cells are susceptible to AICD induced through TCR-mediated recognition of allogeneic MHC class II molecules[52,53]. Our data demonstrated that autoantigen stimulation results in a significant decrease in anti-Fas-induced $CD4^+$ T cell apoptosis in a dose-dependent manner. In addition, AICD is triggered in $CD4^+$ T cells by a specific antigenic peptide, e.g. tetanus toxoid or myelin basic protein, presented by the appropriate MHC class II molecules (54), supporting the notion that AICD can be triggered in activated cells through the TCR-mediated recognition of antigen. The specificity of cytotoxic T lymphocyte (CTL) 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 as a prelude to organ-specific autoimmune diseases.

Tissue-specific apoptosis induced by estrogen deficiency

Recently, we have evaluated the effects on autoantigen cleavage in estrogen deficient healthy C57BL/6(B6) mice treated with an ovariectomy (Ovx) (Fig. 3A). We have demonstrated a significant apoptosis associated with α-fodrin cleavage in the salivary gland cells of estrogen deficient healthy C56BL/6 (B6) mice[55] (Fig. 3B). These data suggest that α-fodrin cleavage triggered by estrogen deficiency plays a role in the development of autoimmune exocrinopathy in the salivary and lacrimal glands. In contrast, apoptotic cells in the salivary glands were not found in ERαKO mice. In *in vitro* studies using primary cultured mouse salivary gland cells (MSG) and human salivary gland cells (HSG), we found a cleavage product of 120 kDa α-fodrin in cells that had undergone tamoxifen (Tam)-induced apoptosis, but not in other types of cells including MCF-7. Since pretreatment with estrogen inhibits the Tam-induced apoptosis of MSG and HSG cells, estrogen may play a crucial role in the apoptosis-related signal pathway. When we analyzed whether cystein proteases are involved in Tam-induced apoptosis of HSG cells, we observed a time-dependent increase in the active forms of caspase 1. In addition, we found that the promoter activity of caspase 1 was significantly increased when HSG cells transfected with the promoter-caspase 1 gene were stimulated with Tam. Indeed, active forms of caspase 1 were detected only in the salivary gland tissues from Ovx-B6 mice. Of importance is that adoptive transfer of α-fodrin-reactive T cells into Ovx-B6 mice resulted in the development of autoimmune exocrinopathy similar to SS. It has been strongly suggested that α-fodrin

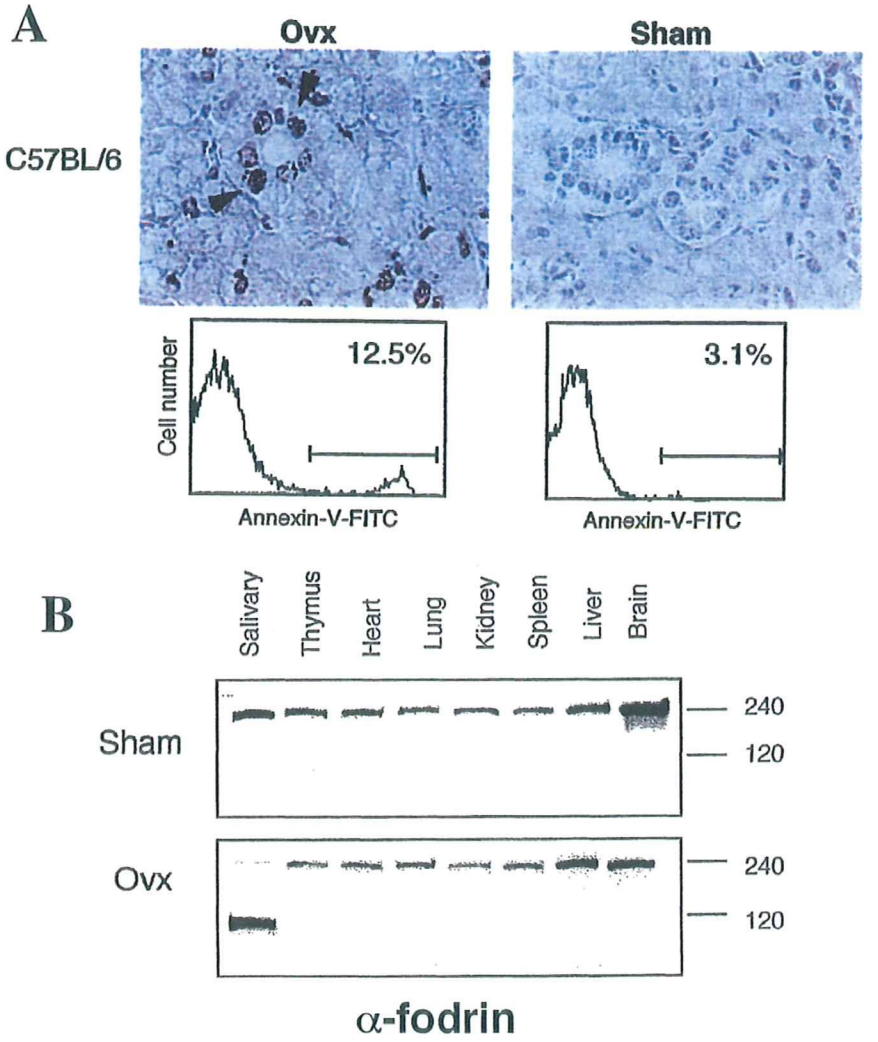

Fig. 3 Tissue-specific apoptosis in the salivary glands induced by estrogen deficiency[55]. (A) Detection of TUNEL$^+$-apoptotic cells in the salivary gland sections from Ovx- and Sham-C57BL/6 (B6) mice at 3 weeks after Ovx (arrows). Annexin V-flow cytometric analysis using MSG cells demonstrated 12.5%-positive cells detected in Ovx-B6 mice, but 3.1%-positive in Sham-B6 mice. Data are represented in triplicate. (B) Detection of α-fodrin cleavage in various tissues from Ovx- and Sham-B6 mice on Western blot analysis showing the distinct band of 120 kDa in the salivary gland tissue alone from Ovx-B6 mice.

fragments induced by estrogen deficiency may play an important role in the development of autoimmune lesions in Sjögren's syndrome[55]. We have demonstrated that antiestrogenic actions, including estrogen deficiency, may have a crucial influence on apoptosis and α-fodrin proteolysis through an increased caspase activity in the salivary gland cells, suggesting a novel mechanism for the development of organ-specific autoimmunity in postmenopausal women. Molecular mechanisms responsible for tissue-specific apoptosis induced by estrogen deficiency are now being investigated.

Apoptosis in labial gland biopsies with Sjögren's syndrome

To evaluate tissue-specific apoptosis and autoantigen cleavage in the salivary glands with Sjögren's syndrome, we analyzed human biopsy specimens by immunohistology and immunoblotting. All patients with SS were female, had documented xerostomia and

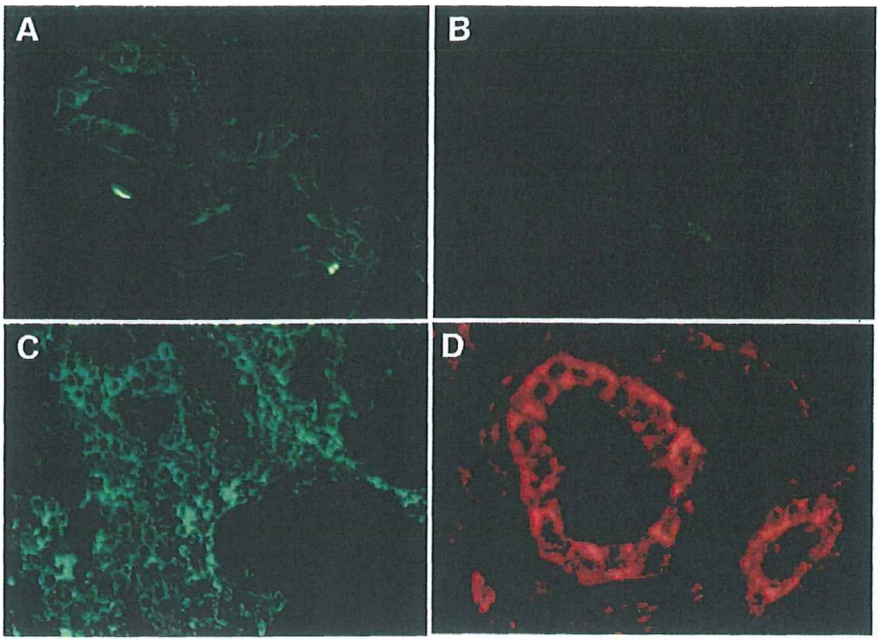

Fig. 4 Immunohistological features in the labial gland biopsies with Sjögren's syndrome. (A) A cleavage product of 120 kDa α-fodrin was present exclusively in epithelial duct cells of the SS salivary glands with Sjögren's syndrome, but not in (B) control salivary glands with mucous cyst. (C) Immunofluorescence analysis revealed that the majority of tissue-infiltrating lymphoid cells bore FasL, and (D) epithelial duct cells stained positively with Fas on their cell surface in SS salivary glands with Sjögren's syndrome. Six samples for each were examined.

keratoconjunctivitis sicca, and fulfilled the San Diego criteria for the diagnosis of Sjögren's syndrome. Analyses were performed under the certification of the ethics board of Tokushima University Hospital. Immunofluorescence analysis using polyclonal Ab against synthetic 120 kDa α-fodrin 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 (Fig. 4A & 4B). Western blot analysis confirmed the same results, indicating that a cleavage product of 120 kDa α-fodrin is present in the diseased glands with Sjögren's syndrome. Immunohistochemical analysis revealed that a majority of infiltrating cells were $CD4^+$, and that a small number of $CD8^+$ cells were present in the salivary glands with Sjögren's syndrome. Immunofluorescence analysis revealed that a large number of infiltrating lymphoid cells bear FasL in the salivary glands with Sjögen's syndrome (Fig. 4C), and epithelial duct cells stained positively with Fas on their cell surface (Fig. 4D). Thus, we provided evidence suggesting that Fas/FasL-mediated apoptosis may be involved in *in vivo* cleavage of α-fodrin autoantigen in the salivary glands with Sjögren's syndrome.

On the horizon

A cleavage product of 120 kDa α-fodrin was identified as an important organ-specific autoantigen in human Sjögren's syndrome. The data discussed in this review are strongly suggestive of the essential role of caspase cascade for α-fodrin autoantigen cleavage leading to tissue destruction in autoimmune exocrinopathy in Sjögren's syndrome. Alpha-fodrin cleavage by caspases can potentially lead to cytoskeletal rearrangement, and it is of interest to point out that α-fodrin binds to ankylin, which contains a cell death domain[56]. It has been shown that cleavage products of α-fodrin inhibit ATP-dependent glutamate and γ-aminobutyric acid accumulation into synaptic vesicles[57], suggesting that a cleavage product of 120 kDa α-fodrin could be a novel component of unknown immunoregulatory networks such as cytolinker proteins[58]. *In vitro* T cell apoptosis assay indicated that FasL-mediated AICD is down-regulated by autoantigen stimulation in spleen cells

from murine Sjögren's syndrome. The processing of sFasL occurs in autoantigen-specific $CD4^+$ T cells *in vivo*, and a significant increase in expressions of MMP-9 mRNA was observed in spleen cells from the mouse model. These data indicate that the increased generation of sFasL inhibits the normal AICD process, leading to the proliferation of effector $CD4^+$ T cells. We have recently observed that $CD4^+$ T cells freshly isolated from PBMC with SS patients induce Th1 cytokine (IL-2, IFN-γ), suggesting that the autoantigen peptide plays an important role in the Th1/Th2 balance *in vivo*. Moreover, α-fodrin peptide-pulsed $CD4^+$ T cells down-regulate Fas-mediated apoptosis when pulsed with each corresponding peptide. Although antigen-induced T cell death is known to be regulated by CD4 expression, molecular mechanisms responsible for T cell death should be further elucidated. However, it remains unclear whether T cells specific for endogenous epitopes play a significant pathologic role in tissue damage during clinical episodes. Our data demonstrated that AFN peptide-stimulation results in a significant decrease in anti-Fas-induced $CD4^+$ T cell apoptosis. 120 kDa α-fodrin, the apoptosis associated breakdown product, may play an important role in the development of SS, and that the autoantigen peptide is a novel participant in the down-modulation of Th1/Th2 balance and peripheral T cell tolerance. It is a future possibility that a peptide analogue of autoantigen could be used as an immunotherapeutic agent.

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