

REVIEW

The role of caspase cascade on the development of primary Sjögren's syndrome

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Abstract: Primary Sjögren syndrome (SS) is an autoimmune disease characterized by diffuse lymphoid cell infiltrates in the salivary and lacrimal glands, resulting in symptoms of dry eye and dry mouth due to insufficient secretion. Previously, we have identified the 120 kDa α -fodrin as an important autoantigen on the development of SS in both animal model and SS patients, but the mechanism of α -fodrin cleavage leading to tissue destruction in SS remains unclear. In murine primary SS model, tissue-infiltrating CD4⁺ T cells purified from the salivary glands bear a large proportion of Fas ligand (FasL), and the salivary gland duct cells constitutively possess Fas. Infiltrating CD4⁺ T cells identified significant ⁵¹Cr release against mouse salivary gland (MSG) cells. *In vitro* studies demonstrated that apoptotic MSG cells result in a specific α -fodrin cleavage into 120 kDa, and preincubation with caspase-inhibitor peptides blocked α -fodrin cleavage. The treatment with caspase-inhibitors *in vivo* prevented the development of autoimmune lesions in the salivary and lacrimal glands. Thus, an increased activity in caspase cascade may be involved in the progression of α -fodrin proteolysis and tissue destruction on the development of SS.

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INTRODUCTION

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 (RNP) particles SS-A/Ro and SS-B/La (1-4). The spectrum of presentation of the disease is broad, ranging from the organ-localized dysfunction of exocrine gland to systemic complications such as liver, kidney and lung involvement (5). Although it has been assumed that a combination of immunologic, genetic, and environmental factors may play a key role on the development of autoimmune

lesions, little is known about the disease pathogenesis. Autoimmune diseases are characterized by tissue destruction and functional decline due to autoreactive T cells that escape self-tolerance (6, 7). Although the specificity of cytotoxic T lymphocyte (CTL) function has been an important issue of organ-specific autoimmune response, the mechanisms responsible for tissue destruction in SS remain to be elucidated. The histopathological changes in the minor salivary gland biopsy are characterized by focal and/or diffuse lymphoid cell infiltrates and parenchymal destruction. The majority of lymphoid cells in the salivary biopsy are CD4⁺ T cells with a small proportion of CD8⁺ T cells (2). These T cells express the $\alpha\beta$ antigen receptor and cell surface antigens associated with mature memory T cells. Since it was evident a preferential use of specific variable region segments of the antigen receptor β chain by salivary gland T cells (8), it has been assumed that a unknown organ-specific autoantigen

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targeted by autoreactive T cells may be present in the salivary glands. We have established and analyzed an animal model for primary SS in NFS/*sld* mutant mice thymectomized 3 day after birth (3d-TX) (9-20). When the repertoire of T cell receptor (TCR) V β genes transcribed and expressed within the inflammatory infiltrates was analyzed in an animal model, a preferential utilization of TCR V β gene was detected in these lesions from the onset of disease (10). We have previously identified a 120 kDa organ-specific autoantigen from the salivary gland tissues of this animal model (21). The sequence of the first 20 NH₂-terminal residues was found to be identical to that of cytoskeletal protein human α -fodrin (21). Furthermore, sera from patients with SS reacted positively with purified 120 kDa antigen, and proliferative response of peripheral blood lymphocytes (PBMC) from SS patients to the purified autoantigen was detected, but not from SLE or RA patients, and healthy controls. These results indicate that the anti-120 kDa α -fodrin immune response plays an essential role on the development of primary SS. Recent reports have demonstrated evidences that caspase 3 is required for α -fodrin cleavage during apoptosis (22-24). In Jurkat cells, caspase 3-like proteases have been reported to cleave α -fodrin and poly (ADP-ribose) polymerase (PARP) but with differential sensitivity to the caspase 3 inhibitor, DEVD-CHO (24). We speculate that an increase in the enzymatic activity of apoptotic proteases is involved in the progression of α -fodrin proteolysis during development of SS.

Involvement of Fas and FasL in tissue destruction

It is now clear that the interaction of Fas with FasL regulates a large number of pathophysiological process of apoptosis (25, 26). We speculate that an increase in the enzymatic activity of apoptotic proteases is involved in the progression of α -fodrin proteolysis during development of SS. To determine the possible involvement of Fas and FasL in tissue destruction of SS, we first analyzed Fas expression in the salivary gland specimens of 3d-thymectomized (3d-Tx) NFS/*sld* mouse model (10) and in the mouse salivary gland cells (MSG) isolated from non-thymectomized (non-Tx) NFS/*sld* mice. Immunohistology revealed that the majority of tissue-infiltrating lymphoid cells in the salivary glands bear FasL in SS model, and epithelial duct cells express Fas antigen on their

cell surface. We found that tissue-infiltrating CD4⁺ T cells isolated from the affected glands bear a large proportion of FasL (>85%), compared with CD8⁺ T cells bearing FasL on flow cytometry (<23%) (P<0.01) (Fig. 1A). A minor proportion of infiltrating CD4⁺ T cells express Fas (<31%), and CD8⁺ T cells bearing Fas were negligible (<5%). Primarily cultured MSG cells isolated from 3d-Tx, non-Tx NFS/*sld* and C57BL/6 mice constitutively express Fas with high proportion (51%-60%) on flow cytometry (Fig. 1B). Immunohistochemically, epithelial duct cells in non-Tx NFS/*sld* and C57BL/6 salivary glands are positive for Fas. RT-PCR analysis demonstrated that Fas mRNA was constitutively present in the salivary glands of SS model, non-Tx NFS/*sld*, and normal C57BL/6 mice. MSG cells isolated from these mice did not express FasL on flow cytometric analysis. A significant increase of TUNEL⁺-apoptotic epithelial duct cells in the salivary glands was observed in SS model mice, compared with those in non-Tx NFS/*sld*, and C57BL/6 mice at all ages. We next investigated whether tissue-infiltrating T cells are responsible for tissue destruction as judged by *in vitro* ⁵¹Cr release cytotoxic assay against MSG cells. Infiltrating CD4⁺ T cells, but not CD8⁺ T cells, identified significant ⁵¹Cr release against MSG cells. These cytotoxic activities were almost entirely inhibited by incubation with anti-murine neutralizing FasL mAb (FLIM58 : 1 μ g/ml), indicating that the cytotoxicity by activated CD4⁺ T cells towards salivary gland epithelial cells was Fas-based.

Participation of caspases in α -fodrin cleavage

To confirm the organ-specificity of a cleavage product of α -fodrin, we investigated various strains of mice with salivary gland destruction, such as MRL/*lpr*, nonobese diabetic (NOD) mice, in addition to 3d-TX NFS/*sld* mice. Protein immunoblot analysis demonstrated that the 120 kDa α -fodrin was detected in these affected glands, but not in normal mice. We examined the *in vitro* cleavage of α -fodrin using 240 kDa α -fodrin in MSG cells. Anti-Fas Ab-induced apoptosis was confirmed by FACS analysis using *in situ* TUNEL procedure, and DNA laddering and formation. We could detect the 120 kDa α -fodrin in apoptotic MSG cells on immunoblotting. We examined the *in vitro* cleavage of α -fodrin in MSG cells induced by anti-Fas mAb (Jo2 : 300 ngml⁻¹). Anti-Fas mAb-stimulated apoptosis in MSG cells was con-

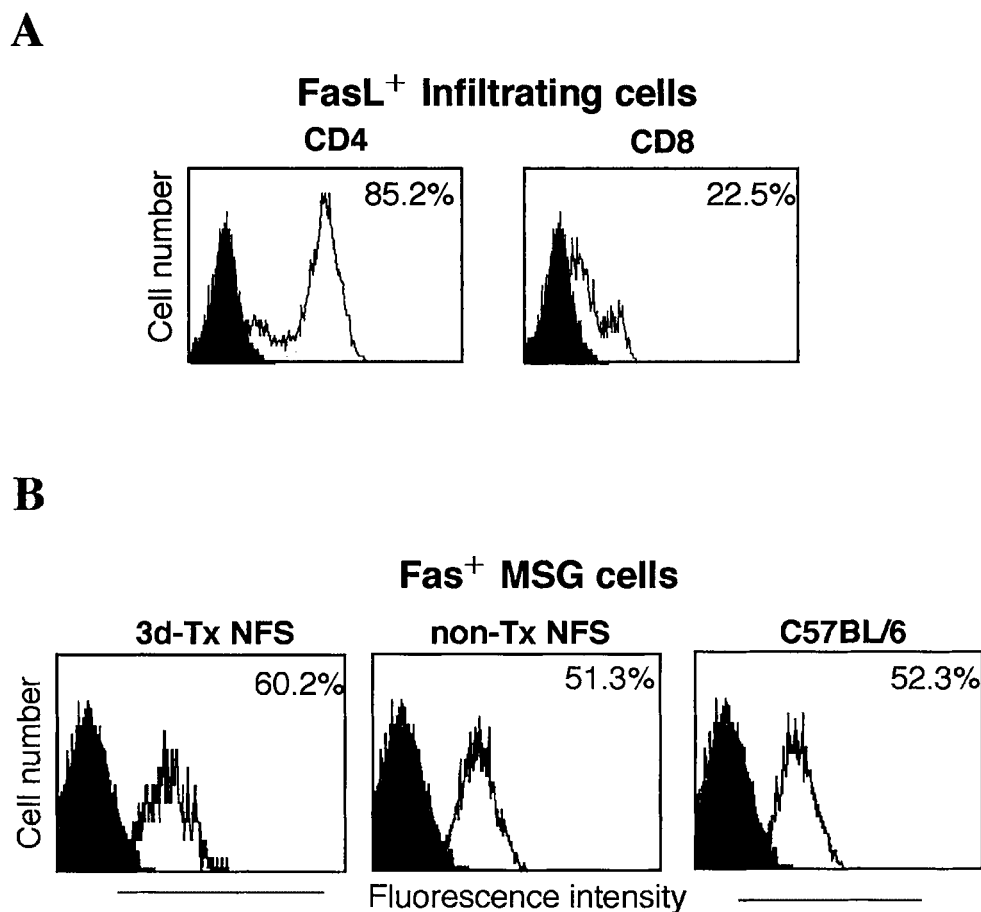


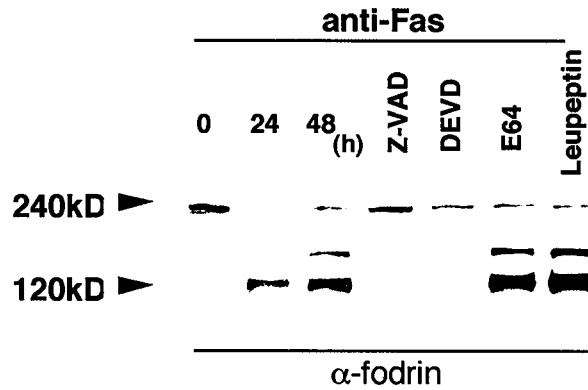
Figure 1. FasL and Fas expression in the salivary gland tissues from 3d-Tx NFS/*sld* mice. (A) Flow cytometric analysis of FasL expression on tissue-infiltrating lymphocytes isolated from salivary glands of 3d-Tx NFS/*sld* mice gated on CD4, and CD8. FasL expression on tissue-infiltrating CD4⁺ T cells was prominent compared with that on CD8⁺ T cells. Five mice in each group were analyzed at 8-, and 12-wk-old of age. (B) Flow cytometric analysis of Fas expression on MSG cells from 3d-Tx, non-Tx NFS/*sld*, and normal C57BL/6 mice. Fas expression was constitutively observed on MSG cells from each group of mice. Five mice in each group were analyzed.

firmed by flow cytometry of 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 on time-dependent manner, and the cleavage was entirely blocked by preincubation with caspase inhibitors (z-VAD-fmk, DEVD-CHO) (Fig. 2A). Protease inhibitor cocktails, cysteine protease inhibitors (E 64), and serine protease inhibitor (Leupeptin) had no significant effect on 120 kDa α -fodrin cleavage in apoptotic MSG cells (Fig. 2A). The 113 kDa PARP in apoptotic MSG cells was not cleaved to smaller fragments. We next investigated whether cysteine proteases are involved in α -fodrin cleavage on apoptotic MSG cells. The caspase 1- and caspase 3-like activities in anti-Fas mAb-stimulated MSG cell extracts were determined using fluorescent substrates (27), and caspase inhibitors (z-VAD-fmk, DEVD-CHO) inhibited these activities at different dose (0.2, 2, and 20 μ M) (Fig. 2B).

Preventive effect of caspase inhibitors in vivo

We next examined whether α -fodrin cleavage to 120 kDa fragment on apoptotic human salivary gland cells (HSG) (28) could be blocked by preincubation with specific protease inhibitors. In apoptotic HSG cells, calpain inhibitor peptide and caspase inhibitor (Z-VAD-fmk) had partially blocked 120 kDa α -fodrin formation. Moreover, a combination of calpain inhibitor peptide and caspase inhibitors (Z-VAD-fmk and Z-DEVD-fmk) almost entirely inhibited the formation of 120 kDa α -fodrin. Protease inhibitor cocktails, other cysteine protease inhibitors (E64), and serine protease inhibitor (Leupeptin) had no effect on 120 kDa α -fodrin cleavage in apoptotic HSG cells. By immunohistochemistry using polyclonal Ab against synthetic 120 kDa α -fodrin, a cleavage product of α -fodrin was present exclusively in epithelial duct cells of the labial salivary gland biopsies from SS patients, but not in control individuals. Protein

A



B

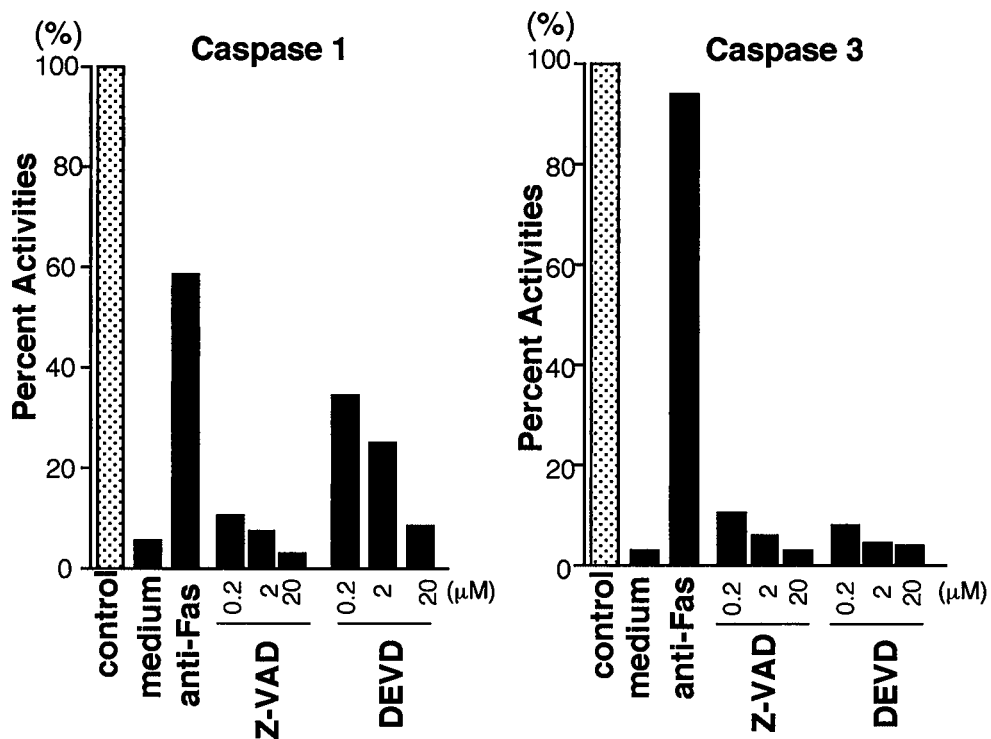


Figure 2. (A) Western blot analysis demonstrated that the 240 kDa α -fodrin in apoptotic MSG cells was cleaved to smaller fragments into 120 kDa on time-dependent manner, and the cleavage product was entirely blocked by preincubation with caspase inhibitors (z-VAD-fmk, DEVD-CHO) measured at 24 h. Protease inhibitor cocktails, cysteine protease inhibitors (E64), and serine protease inhibitor (Leupeptin) had no significant effect on α -fodrin cleavage. (B) Activation of caspase 1-like and caspase 3-like proteases was detected during anti-Fas-induced apoptosis, and caspase inhibitors inhibited these activities. Cytosolic extracts were prepared from MSG cells (1×10^7 cells) which were treated at 37 $^{\circ}$ C with 300 ng/ml¹ of Jo-2. 100% activity as control was calculated using the values that 300U/ml recombinant caspase 1 or caspase 3 was added to each substrate (200 μ M MOCAC-YVAD(dnp)-NH₂ and MOCAC-DEVD (dnp)-NH₂).

immunoblot analysis confirmed the same results. This indicates that a cleavage product of 120 kDa α -fodrin is present in the diseased glands with human SS, but not in control glands. We further investigated whether the i.v. injection of caspase-inhibitors protects SS animal model against the development of autoimmune lesions. The both treatment with i. v. injection of z-VAD-fmk and DEVD-CHO (3 times

per week) ($P < 0.005$) prevented the development of autoimmune lesions in the salivary and lacrimal glands. The average saliva and tear volume of the treated SS animal model was significantly higher than that of the control group. A significant decrease of autoantigen-specific T cell proliferation was observed in spleen cells from treated mice. In addition, serum autoantibody production against

120 kDa α -fodrin was clearly inhibited by the treatment with caspase-inhibitors. The treatment of murine SS model with i.v. injection of z-VAD-fmk and DEVD-CHO prevented the development of autoimmune conditions, resulting in restoration of saliva and tear secretion. These results suggest that increased activity of caspase cascade is involved in the progression of α -fodrin proteolysis during the initial stages on the development of primary SS.

Autoimmune lesions induced by immunization with autoantigen

To examine the autoimmune nature of 120 kDa α -fodrin, recombinant α -fodrin protein identical to an autoantigen was administered subcutaneously (s.c.) into normal NFS/*sld* mice at 4 wks. Organ-specific autoimmune lesions similar to SS developed at 8 wks after the injection in almost all mice immunized with autoantigen, but not in all groups of control (19). No inflammatory lesions were observed in other organs. A majority of infiltrating cells were CD4⁺ and FasL⁺, and the epithelial duct cells express Fas on their cell surface. A specific cleavage of α -fodrin into 120 kDa was detected in the salivary glands of immunized mice, but not in controls. Mice injected with recombinant autoantigen showed a significant increase of autoantigen-specific T cell proliferation in spleen cells. A high titer of serum autoantibodies against 120 kDa α -fodrin was detected in immunized mice, compared with control mice by ELISA. These data demonstrated evidences that a cleavage product of 120 kDa α -fodrin is pathogenic autoantigen on the development of murine primary SS.

Concluding remarks

There is increasing evidences that the cascade of caspases is a critical component of the cell death pathway (29-31), and a few proteins have been found to be cleaved during apoptosis. We provided evidence that α -fodrin is cleaved by one or more members of caspases during apoptotic cell death in SS salivary glands. 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 (32). It has been shown that cleavage products of α -fodrin inhibit ATP-dependent glutamate and γ -aminobutyric acid accumulation into synaptic vesicles (33), suppos-

ing that a cleavage product of 120 kDa α -fodrin could be a novel component of an unknown immunoregulatory networks such as cytolinker proteins (34). These results are strongly suggestive of essential roles of caspase cascade for α -fodrin cleavage leading to tissue destruction in autoimmune exocrinopathy of primary SS.

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