Involvement of Apoptotic Protease Cascade for Tissue Destruction in Sjögren’s Syndrome

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Abstract. Sjögren syndrome (SS) is an autoimmune disease characterized by diffuse lymphoid cell infiltrates in the salivary and lacrimal glands, resulting in symptoms of dry mouth and eyes due to insufficient secretion. Although it has been assumed that a combination of immunologic, genetic and environmental factors may play a key role in the development of autoimmune lesions in the salivary and lacrimal glands, little is known about the disease pathogenesis of SS in humans. We have identified the 120 kDa α-fodrin as an important autoantigen in the development of SS in both an animal model and SS patients, but the mechanism of α-fodrin cleavage leading to tissue destruction in SS remains unclear. Tissue-infiltrating CD4+ T cells purified from the salivary glands of a mouse model for SS bear a large proportion of Fas ligand and the salivary gland duct cells possess apoptotic receptor Fas. Anti-Fas antibody-induced apoptotic salivary gland cells result in specific α-fodrin cleavage to the 120 kDa fragment in vitro. Preincubation with a combination of calpain and caspase inhibitor peptides could be responsible for inhibition of the 120 kDa α-fodrin cleavage. Thus, an increase in apoptotic protease activities including calpain and caspases may be involved in the progression of α-fodrin proteolysis and tissue destruction in the development of SS.

Key words: Sjögren’s syndrome; autoantigen; protease; apoptosis.

Introduction

Sjögren’s syndrome (SS) is a chronic autoimmune disorder affecting the salivary and lacrimal glands and leads to the clinical symptoms of dryness of the mouth and eyes (sicca syndrome)7. The histopathological changes in the minor salivary gland biopsies are characterized by focal and/or diffuse lymphoid cell infiltrates and parenchymal destruction. The majority of lymphoid cells in the salivary biopsies are CD4+ T cells, with a small proportion of CD8+ T cells11. These T cells express the αβ antigen receptor and cell surface antigens associated with mature memory T cells. Since a preferential use of specific variable region segments of the antigen receptor β chain by salivary gland T cells was evident12, it has been assumed that an unknown organ-specific autoantigen targeted by autoreactive T cells may be present in the salivary glands.

We have established an animal model for primary SS in NFS/sld mutant mice thymectomized 3 day after birth (3d-Tx)14, 16. When the repertoire of T cell receptor (TCR) Vβ genes transcribed and expressed within the inflammatory infiltrates was analyzed in the animal model, a preferential utilization of TCR Vβ gene was detected in these lesions from the onset of disease14. We have recently identified a 120 kDa organ-specific auto-

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antigen from the salivary gland tissues of this animal model. The sequence of the first 20 NH2-terminal residues was found to be identical to that of cytoskeletal protein human α-fodrin. 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 systematic lupus erythematosis (SLE) or rheumatoid arthritis (RA) patients and healthy controls. These results indicate that the anti-120 kDa α-fodrin immune response plays an essential role in the development of primary SS.

Autoimmune diseases are characterized by tissue destruction and functional decline due to autoreactive T cells that escape self-tolerance. Although the specificity of the 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. It is now clear that the interaction of Fas with Fas ligand (FasL) regulates a large number of the pathophysiological processes of apoptosis. We speculate that an increase in the enzymatic activity of apoptotic proteases is involved in the progression of α-fodrin proteolysis during the development of SS.

Involvement of Fas and FasL in Tissue Destruction

To determine the possible involvement of Fas and FasL in the tissue destruction of SS, we first analyzed Fas expression in the salivary gland specimens of the 3d-Tx NFS/ld mouse model and in the mouse salivary gland cells (MSG) isolated from non-thymectomized (non-Tx) NFS/ld mice. Immunohistochemistry revealed that epithelial duct cells express a large amount of Fas on their cell surface in 3d-Tx NFS/ld mice, also detected by in situ TUNEL assay. However, we found that epithelial duct cells in non-Tx NFS/ld and normal salivary glands are constitutively positive for Fas, but not for TUNEL staining, suggesting that Fas expression does not correlate with its apoptotic function in normal salivary gland cells. Isolated MSG cells express Fas with high proportion (>66%) on FACS staining (Fig. 1). We found that the tissue-infiltrating CD4+ T cells purified from the salivary gland tissues of 3d-Tx NFS/ld mice bear a large proportion of FasL (>73%) (Fig. 1). Immunohistochemical analysis revealed that FasL expression was completely absent on the salivary gland epithelial cells in any strain of mouse. RT-PCR analysis demonstrated that Fas mRNA was present in both salivary glands, and a high level of FasL mRNA was detected in the salivary glands of 3d-Tx NFS/ld mice, but not in non-Tx and normal mice. Protein immunoblot analysis confirmed the expression of FasL in salivary glands of 3d-Tx NFS/ld mice.

We next investigated whether activated CD4+ T cells are responsible for tissue destruction as judged by in vitro 51Cr release cytotoxic assay against MSG cells. MSG cells (2 × 10^6) in 7.5 ml of MEM were labeled overnight at 37°C in 5% CO2 with 300 μCi of sodium [51Cr]-chromate. CD4+ and CD8+ T cells purified from splenocytes using magnetic beads were incubated with ConA and recombinant human IL-2. The splenic CD4+ T cells activated with ConA and IL-2 showed significant 51Cr release against MSG cells (Fig. 2). These cytotoxic activities were almost entirely inhibited by incubation with anti-murine neutralizing FasL antibody, indicating that the cytotoxicity by activated CD4+ T cells towards salivary gland epithelial cells was FasL-based.

Participation of Calpain and 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 non-obese diabetic (NOD) mice, in addition to 3d-TX NFS/ld 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 antibody (Ab)-induced apoptosis was confirmed by FACS analysis using the
Fig. 2. Cytotoxic activity of activated CD4+ T cells from spleen towards Fas-sensitive MSG cells. Spleenic CD4+ T cells activated with ConA and IL-2 identified significant $^{51}$Cr release against MSG cells. This activity was almost entirely inhibited by anti-murine neutralizing FasL antibody.

Fig. 3. Protein immunoblot analysis of 120 kDa α-fodrin in apoptotic human salivary gland (HSG) cells treated with anti-Fas mAb. 120 kDa α-fodrin was detected in apoptotic HSG cells.

in situ TUNEL procedure and by DNA laddering and formation. We could detect the 120 kDa α-fodrin in apoptotic MSG cells on immunoblotting. Similar evidence was obtained with a human salivary gland (HSG) epithelial cell line. Apoptotic HSG cells induced by treatment with anti-Fas Ab was confirmed. Immunoblot analysis demonstrated that the 240 kDa α-fodrin on apoptotic HSG cells was cleaved to smaller fragments, including 150 kDa and 120 kDa (Fig. 3).

We next investigated whether cysteine proteases are involved in α-fodrin cleavage on apoptotic HSG cells. Anti-Fas Ab treated HSG cells were positive for mAb to µ-calpain, and to ICE (p20) and CPP32 in association with apoptosis. The ICE- and CPP32-like activities in anti-Fas Ab treated HSG cell extracts were then determined using fluorescent substrates. The specific inhibitor for ICE (Ac-YVAD-cmk) or CPP32 (Z-DEVD-fmk) was added to the reaction mixture at a concentration of 1 µM. Specific ICE- and CPP32-like activities were determined by subtracting the values obtained in the presence of inhibitors. These results suggest that protease families including calpain and caspases participate in the progression of α-fodrin cleavage of apoptotic HSG cells.

Preventive Effect of Caspase Inhibitor in Vivo

To address this hypothesis, we examined whether α-fodrin cleavage to the 120 kDa fragment on apoptotic HSG cells 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 (E-64) and serine protease inhibitor (Leupeptin) had no effect on 120 kDa α-fodrin cleavage in apoptotic HSG cells. The specificity of a combination of calpain and caspase inhibitors towards the decrease in 120 kDa α-fodrin in the HSG cells will be further quantified in detail.

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 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 next investigated whether the intravenous injection of caspase inhibitor, Z-VAD-fmk, protects animals against the development of autoimmune lesions. The treatment with intravenous injection of Z-VAD-fmk prevented the development of autoimmune conditions including lymphocytic infiltration and auto-antibody production to the whole 120 kDa molecule.
Perspective

Although cleavage of certain autoantigens during apoptosis may reveal immunocryptic epitopes that could potentially induce autoimmune responses in systemic autoimmune diseases\(^4\),\(^3\), no evidence whether \textit{in vivo} cleavage of autoantigen occurs in organ-specific autoimmune diseases has yet been described. The roles of the Fas/FasL system in the pathogenesis of autoimmune disease have already been proposed\(^1\). 25, 26, 28, 30, 33. It has been recently 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\(^13\). In contrast, expression of Fas by pancreatic \(\beta\) cells has been shown to have a major influence on the susceptibility of tissue destruction in NOD mice to diabetes\(^2\). In this study, we provide evidence suggesting that Fas/FasL-mediated apoptosis may be involved in the initial cascade of the \textit{in vivo} cleavage of autoantigen and that CD4\(^+\) cytotoxic T cells may participate in the tissue destruction of SS salivary glands.

When human T cell leukemia CEM cells were induced to undergo apoptosis, the 240 kDa \(\alpha\)-fodrin was cleaved to a single detectable fragment of 120 kDa\(^27\). It was plausible that the 120 kDa fragment is a break-down product of the 150 kDa \(\alpha\)-fodrin cleavage\(^22\). The analysis of proteolytic events associated with apoptosis is to define the mechanisms leading to protease activation and to identify key substrates whose cleavage might be linked to the profound changes in cellular architecture. There is increasing evidence that calpain is overactivated in autoimmune conditions and subsequent tissue destruction\(^13\),\(^24\). Moreover, the cascade of caspases is a critical component of the cell death pathway\(^17\),\(^18\),\(^20\),\(^29\) and a few proteins have been found to be cleaved during apoptosis. These include poly (ADP-ribose) polymerase (PARP), a small U1 nuclear ribonucleoprotein (RNP), and \(\alpha\)-fodrin, which were subsequently identified as substrates for caspases\(^8\). We provided evidence that \(\alpha\)-fodrin is not only cleaved by calpain, but also by one or more members of the caspases during Fas/FasL mediated-apoptosis in SS salivary glands. Fodrin cleavage by calpain and caspases can potentially lead to cytoskeletal derangement. Above all, it is of great interest to point out that \(\alpha\)-fodrin binds to ankylin, which contains a cell death domain\(^9\).

In conclusion, these results are strongly suggestive of a role for calpain- and caspase-mediated cleavage of \(\alpha\)-fodrin in the functional activation of autoreactive T cells in SS, and could be consistent with a role for Fas/FasL-mediated apoptosis in the development of autoimmune tissue destruction. Moreover, \textit{in vivo} preventive effects against autoimmune lesions treated with protease inhibitors have important implications for testing useful therapies.

References


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