Activated caspase 3 and cleaved poly(ADP-ribose)polymerase in salivary epithelium suggest a pathogenetic mechanism for Sjögren’s syndrome


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Abstract

Objective. Apoptosis is an organized energy-dependent process of cellular self-destruction carried out by proteolytic enzymes such as the caspases. These enzymes may play a role in epithelial cell apoptosis in Sjögren’s syndrome (SS). A classical caspase substrate is poly(ADP-ribose)polymerase (PARP), a DNA repair enzyme. To elucidate the molecular mechanisms responsible for salivary gland dysfunction in SS, we studied the expression of caspase and PARP in SS salivary gland biopsies.

Methods. The presence of activated caspases (caspases 3 and 9) and cleaved PARP (85 kDa) in SS biopsies was demonstrated by immunohistochemistry using specific polyclonal antibodies.

Results. Initial studies performed with an antibody reagent that recognizes both active and inactive forms of caspase 3 identified this enzyme in SS salivary ductal and acinar cells. Activated caspase 3 and cleaved PARP were strongly expressed in ductal and acinar cells in SS salivary glands (13–15). Ductal and acinar cells from normal salivary glands (n = 5) stained with less intensity compared with SS tissue. Staining for activated caspase 9 was negative in all samples. Likewise, infiltrating lymphocytes were negative for caspase 3, caspase 9 and cleaved PARP.

Conclusion. This study shows that caspase 3 is important in the salivary dysfunction of SS, while caspase 9 appears not to be involved.

Key Words: Sjögren’s syndrome, Apoptosis, Salivary gland, Caspase, CD95, Epithelial cells.

Primary Sjögren’s syndrome (SS) is an autoimmune rheumatic disease characterized by xerostomia, xerophthalmia and xerocrine gland hypofunction [1–3]. The glands are infiltrated by lymphocytes that consist predominantly of mature T and B cells [4]. This disease occurs in 2–4 million US Americans [5]. Recent European studies have reported the prevalence of SS to range from 0.6 to 3% of the general population [6–9].

There is a growing body of evidence indicating that the SS epithelial acinar cells of the salivary and lachrymal glands undergo apoptosis, although this has been disputed [10]. Studies have shown that apoptotic death in the SS salivary gland may be mediated through the tumour necrosis factor z (TNF-z) receptor [11], CD95 [12–14] or the perforin pathway [15]. In addition, there is an increasing amount of data suggesting that the epithelial cells are active participants in the initiation of the inflammatory process [16–18].

Studies performed over recent years have demonstrated that proteases play critical roles in the initiation and execution of apoptosis. The caspases, a family of cysteine-dependent, aspartate-directed proteases, are prominent among apoptosis-associated molecules [19]. Caspases are synthesized as relatively inactive zymogens (i.e. proenzymes) that become activated by scaffold-mediated transactivation or by cleavage via upstream proteases. Regulation of caspase activation and activity occurs at several levels: (i) zymogen gene transcription; (ii) anti-apoptotic members of the bcl-2 family and other cellular proteins; and (iii) certain cellular inhibitor of apoptosis proteins that can bind to and inhibit active caspases. Once activated, caspases

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cleave a variety of intracellular polypeptides, including major structural elements of the cytoplasm and nucleus, components of the DNA repair machinery and a number of protein kinases. Collectively, these scissions disrupt survival pathways and disassemble important architectural components of the cell, contributing to the morphological and biochemical changes that characterize apoptotic cell death.

We have reported previously that caspase 3 is abnormally elevated in the SS salivary epithelial cell [20]. Here we show that caspase 3 is activated in the salivary epithelial cells of SS patients. This shows that the enzyme participates in the disease process in the SS salivary gland and provides a target for pharmacological intervention.

Patients and methods

Patients

Patients were diagnosed with SS according to the European Community criteria [21]. Biopsy material from SS patients (n = 15) was obtained from the Salivary Dysfunction Clinic (Baylor College of Dentistry, Dallas, TX, USA) and embedded in OCT (Fisher Scientific, Pittsburgh, PA, USA). SS biopsies were graded on a scale of 1–4 according to Chisholm (Fisher Scientific, Pittsburgh, PA, USA). SS biopsies were graded on a scale of 1–4 according to Chisholm [22]. Autopsy material from frozen normal salivary glands (n = 5) was obtained from the National Disease Research Interchange (Philadelphia, PA, USA).

Antibody reagents

Immunohistochemistry was performed with rabbit polyclonal antibodies to active caspase 3 (Pharmingen, San Diego, CA, USA), cleaved poly(ADP-ribose)polymerase (PARP) 89 kDa (Cell Signaling Technology, Beverly, MA, USA), caspase 9 37 kDa (Cell Signaling Technology) and cleaved caspase 9 (p10 subunit) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Immunohistochemistry

Frozen sections (4 μm) were air-dried, fixed in 4% paraformaldehyde for 10 min for cleaved PARP or in cold acetone for 4 min for active caspase 3, and washed in phosphate-buffered saline (PBS; 0.2 M, pH 7.4). Endogenous peroxidase was quenched with 1% H₂O₂ in PBS for 30 min. Afterwards, all the washes were performed in TNT wash buffer (0.5 M Tris–HCl, 0.15 M NaCl, 0.05% Tween 20, pH 7.6). Non-specific binding was inhibited using TNB blocking buffer (0.5 M Tris–HCl, 0.15 M NaCl, 0.5% blocking reagent, pH 7.6) and blocking reagent from (NEN, Boston, MA, USA). Antibodies to active caspase 3 (1:500), cleaved PARP (1:100), the caspase 9 proenzyme (1:100) and active caspase 9 (1:50) were added to the slides in TNB blocking buffer and incubated for 1 h. Tissue sections were incubated for 45 min with peroxidase-conjugated second antibody (1:100) (Dako, Carpinteria, CA, USA) in blocking buffer. After washing, tissue sections were incubated for 10 min with biotinyl tyramide (1:50) in amplification diluent (NEN), followed by a 30 min incubation with alkaline phosphatase-conjugated streptavidin (1:50) (Dako) for active caspase 3, active caspase 9 and PARP detection. Peroxidase-conjugated streptavidin (1:50) (NEN) was used to detect the caspase 9 proenzyme. All the incubations were done at 30°C. MCF7 cells treated with staurosporine (5 μM) for 5 h served as a positive control for activated caspase 9 [23].

To detect active caspase 3, active caspase 9 and cleaved PARP, colour development was achieved by incubation for 5–10 min with Vector Red (Vector Laboratories, Burlingame, CA, USA) (5 mM in 100 mM Tris–HCl, pH 8.2). The caspase 9 proenzyme was visualized with 3,3′-diaminobenzidine (Sigma, St Louis, MO, USA) (6 mg/ml). The counterstaining was with modified Harris haematoxylin solution (Sigma) for 1–3 s. Excess haematoxylin was washed off with water, and the colour was lightened by treatment with 0.3% NH₄OH for 3 min. The sections were dehydrated in graded alcohol and two xylene washes. Slides were mounted with Permount (Fisher Scientific).

Slides were photographed with a light microscope (Olympus BX40) equipped with a 35 mm camera (Olympus PM40), using Elite Chrome 200 ASA film (Eastman Kodak, Rochester, NY, USA).

Acinar epithelial cells were identified by their broad base with narrowing at the apical part of the cell. Ductal cells were defined by a columnar or cuboid feature [24].

Results

We have reported that the acinar epithelial cells in the SS salivary gland undergo apoptosis [13]. This led us to hypothesize that caspase 3 would be involved in the glandular destruction that occurs in SS. To test this hypothesis, normal (n = 5) and SS (n = 15) salivary glands were stained for the activated form of caspase 3 (Fig. 1). Activated caspase 3 was stained weakly in normal salivary glands. The acinar and ductal cells in salivary glands from patients with SS (13 of 15 cases) stained more strongly for this proteolytic enzyme. In all SS glands studied, activated caspase 3 was expressed on major structural elements of the cytoplasm and a number of protein kinases. Collectively, these scissions disrupt survival pathways and disassemble important architectural components of the cell, contributing to the morphological and biochemical changes that characterize apoptotic cell death.

A classical substrate for caspase 3 is PARP. To show that the activated caspase 3 is operating in the salivary epithelial cells, specific antibody to cleaved PARP was used (Fig. 1). The staining pattern with anti-PARP antibody was similar to that with antibody to activated caspase 3. In 13 of 15 biopsies, acinar and ductal epithelial cells in SS salivary glands stained more intensely for cleaved PARP compared with cells from normal salivary glands.

Caspase 9 is activated downstream of caspase 3 through the apoptosome [25]. Normal and SS salivary epithelial cells stained positive for caspase 9 proenzyme (Fig. 2). However, activated caspase 9 was detected in neither SS nor normal glands.
Discussion

Caspase 3 is an effector caspase that is critical in many apoptotic pathways. Caspase 3 cleaves several key enzymes that are needed for normal cell maintenance, such as the DNA repair enzyme PARP [26]. Here we have shown elevated expression of activated caspase 3 and cleaved PARP in the salivary epithelial cells of SS patients. The purpose of this was to confirm the initial observations that SS salivary epithelial cells undergo apoptosis [11, 13–15]. However, the presence of apoptotic cells in the SS salivary gland has been disputed [10]. Thus, the contribution of apoptosis to the pathogenesis of SS requires further investigation.

It should be noted that epithelial cells undergo a normal process of turnover. This occurs even in the context of inflammation. Thus, new cells are generated to replace cells that are dying. When there is excessive apoptosis, the rate of cell loss exceeds the rate of cell replacement, leading to an overall decrease in salivary epithelial cells. The presence of activated caspases in the SS salivary gland supports the concept that excessive cell death contributes to the pathogenesis of this disease.

The apoptotic death in the SS salivary gland may be mediated through the CD95 pathway. There are two general CD95 pathways that involve the caspase cascade [27]. One involves the activation of caspase 8, leading to direct activation of caspase 3 (i.e. type I cells). The other involves the mitochondrial branch of the caspase cascade that is upstream of caspase 3 activation (i.e. type II cells). Although caspase 3 is activated, caspase 9 is not activated in the SS salivary epithelial cells. On the basis of the limits of immunohistochemical detection, we suspect that the epithelial cells in the salivary gland would favour a type I apoptotic pathway. Alternatively, the expression of activated caspase 3 may inhibit normal signalling pathways in non-apoptotic epithelial cells [28, 29].

Our work in NOD mice and SS patients indicates that CD95/CD95 ligand interaction occurs abnormally in the salivary gland [13, 30]. This leads to unwanted apoptosis of acinar cells and the production of cleaved autoantigens by caspases. The latter are rendered

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immunogenic, leading to autoantibody production [31, 33]. This proposed mechanism has the advantages that (i) the immune system is responding in its customary way to autoantigens generated by apoptosis, and (ii) the primary defect lies with the epithelial cells. Indeed, the SS epithelial cell abnormally secretes cytokines [34–36], expresses adhesion molecules [4, 37, 38] and contains viral sequences [39, 40] that can initiate an autoimmune response. This model, in which the lymphocytes are infiltrating target organs in response to autoantigens arising from inappropriate apoptosis of target cells, may also apply to other organ-specific autoimmune diseases, such as Hashimoto’s disease [41] and insulin-dependent diabetes mellitus [42].

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References


