APOPTOSIS AND CELL PROLIFERATION: THE PARADOX OF SALIVARY GLANDS IN SJÖGREN'S DISEASE

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Abstract

Aim: To assess apoptosis and proliferation in salivary glands of patients with primary Sjögren's syndrome.
Methods: Studies were performed in twenty four minor salivary glands from patients with primary Sjögren's syndrome and an equal number of controls. Apoptosis was studied by immunohistochemistry using monoclonal antibodies anti-Fas, FasL and Caspase 3 and apoptotic features by TUNEL. Proliferation was assessed with monoclonal anti-PCNA and anti-Ki67 antibodies.
Results: All salivary glands from Sjögren's display apoptotic molecules along the epithelia of salivary ducts, and in a smaller amount in acinar tissue. The presence of Caspase 3, Fas/FasL was concordant with the expression of apoptosis by TUNEL. Proliferation markers were encountered in inflammatory emigrant cells, but not in ductal epithelia nor in acini. Control biopsies poorly expressed apoptotic or proliferation markers.
Conclusion: Present data suggests that the ductal epithelial and acinar cells of salivary glands from Sjögren's disease patients exhibit increased apoptosis. Proliferation was mainly observed in infiltrating lymphoid cells. Both events constitute a biological paradox related to the inflammatory process of salivary glands in Sjögren's disease.
Keywords: Apoptosis; cell proliferation; Sjögren disease; Salivary glands; PCNA; Caspase 3.

Resumo

Este estudo avalia a apoptose e a proliferação nas glândulas salivares dos doentes com Síndroma de Sjögren primária.
Métodos: A apoptose foi estudada por imunohistoquímica utilizando anticorpos monoclonais anti-Fas, FasL e Caspase 3 e as características apoptóticas por TUNEL. Os estudos foram executados em vinte e quatro glândulas salivares *minor* de doentes com Síndroma de Sjögren primária e num igual número de controlos. A proliferação foi avaliada com anticorpos monoclonais anti-PCNA e anti-Ki67.
Resultados: Todas as glândulas salivares dos doentes com Sjögren apresentavam moléculas apoptoticas no epitélio dos ductos salivares, e menos no tecido acinar, consequentemente a presença do caspase 3, Fas/FasL eram concordantes com a expressão da apoptose por TUNEL. Os marcadores de proliferação foram encontrados nas células inflamatórias presentes, mas não no epitélio ductal nem nos acinos. A expressão de marcadores de apoptose ou de proliferação nos tecidos das biopsias dos controlos foi escassa.
Conclusão: Os dados actuais sugerem que as células do epitélio ductal e dos acinos das glândulas salivares dos doentes com doença de Sjögren têm aumento da apoptose. A proliferação foi observada principalmente no infiltrado celular linfóide. Em conjunto, estes eventos constituem um paradoxo biológico relacionado com o processo inflamatório das glândulas salivares na Síndrome de Sjögren.

Introduction

The pathogenesis of primary Sjögren's disease is multifactorial but the antigen that drives the autoimmune response is still unknown. The disease causes dry eyes and mouth, vasculitis, and neurological symptoms. Symptoms are the result of immune lymphocytic infiltration in lacrimal and salivary glands.[1]

B-cell hyperactivity, increased levels of B-cell-activating factor/B-lymphocyte stimulator and

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anti-Ro or anti La autoantibodies' production are among the abnormalities of the immune system in Sjögren's disease. The glandular dysfunction results from lymphocytic infiltration around the ductal and acinar epithelium. Infiltrates of activated T cells produce inflammatory cytokines, and these mediators cause tissue damage by cytotoxicity, or induce glandular apoptosis; the apoptotic remains contain intracellular autoantigens that trigger autoantibody production. Another effect of cytokines is the induction of functional quiescence, rather than destructive damage; but in the majority of the patients different cytokines induce lymphocyte proliferation. Since pathophysiology of Sjögren's disease is multifactorial, different environmental factors have been implicated such as HTLV-1 and hepatitis C viruses. However, the possible role of virus in the pathogenesis of Sjögren's is still under investigation.[1-4]

The present study addresses the issue of how apoptosis and proliferation may coexist in salivary glands of patients with primary Sjögren's syndrome.

Material and Methods

Minor salivary gland biopsies from 24 patients with primary Sjögren's disease were studied; patients fulfilled the revised version of the European criteria proposed by the American-European Consensus.[5] There were 17 females and 7 males with a mean age of 47 years (range 37 - 57). All the patients and controls were previously informed about the biopsy and written consent was obtained. An equal number of biopsies from controls without Sögren's disease were included; these samples were obtained during oral surgery for other purpose. The protocol was authorized and monitored by the ethics committee of CBE and the study was conducted according to the World Medical Association's Declaration of Helsinki. Specimens were fixed in formaldehyde and included in paraffin, sectioned at 4 µm thin and stained with haematoxylin & eosin. After microscopic evaluation by two pathologists, biopsies were classified according to the revised multilevel assessment of a cumulative focus score (FS).[6] Biopsies were considered negative in case of absence of inflammatory infiltrates (FS=0). The presence of one or more foci/4 mm^2 was classified as positive in spite of the normal appearance of the adjacent glandular parenchyma (0 < FS < 1). Positive FS biopsies were divided in two additional groups as follows: those with less than two foci/4 mm^2 ($1 \leq FS < 2$) and those with two or more foci (FS ≥ 2).

Unstained tissue sections were used for immunohistochemistry.

Immunohistochemistry. Slides containing 4 µm sections of minor salivary glands were dewaxed, then permeabilized with 0.01% Triton X- 100/PBS and washed three times with PBS. Endogenous peroxidase was blocked with 56°C heated horse serum. After washing, tissues were incubated for 1 hour with anti-FasL (sc-19681 Santa Cruz, Biotechnology, Inc. Santa Cruz.CA), anti-Fas (m3553, Dako Carpinteria,CA), anti-Ki-67 antibody (cat 35--1600, Zymed, San Francisco, CA), and anti-PCNA (cat 13-3900, Zymed). Antibodies were diluted 1:1000 in 10% BFS-PBS. After washings with PBS, bound antibodies were tagged with goat anti-mouse IgG labelled with peroxidase. Colour reaction was induced by 2, 2 diaminobenzydine-0.06% H_2O_2 (Sigma, San Luois, MO). Reaction was stopped with 2N sulphuric acid. The slides were then evaluated by three observers; assays were done in triplicate.

TdT-mediated dUTP nick end labeling (TUNEL) was done according to the manufacturer's instructions (Roche Molecular Biochemicals. Penzberg, Germany). Nuclear stripping was performed by immersing the slides for 5 minutes in 10 mM Tris-HCl, pH 8.0, followed by 15 minutes in 20 µg/ml proteinase K dissolved in the same buffer, and finally the slides were washed with PBS. Elongation of the DNA fragments was performed by incubation for 60 minutes at 37ºC with 75 µl of the reaction mixture [DDW, 10XTdT buffer (30 mM Tris base, 140 mM sodium cacodylate, pH 7.2, 1 mM cobalt chloride, 1 mM DTT); 10% of the final volume], fluorescein-11-dUTP (0.5 mg dissolved in 1 ml of 10 mM Tris-HCl, pH 7.0), and TdT enzyme (0.3 enzyme units/µl). The reaction was terminated by adding the stop solution (300 mM NaCl, 30 mM sodium citrate, pH 8.0).

Results

Lymphocyte infiltrates: Biopsies from Sjögren's disease were classified as FS1=five biopsies, FS<2=six, FS = 2=five and FS >2=eight biopsies. Control biopsies from normal individuals were all negative (FS 0).
Apoptotic features: All minor salivary glands show apoptotic markers. As expected, the Fas receptor

was widely distributed along ductal epithelium and in a lesser amount along acini. No difference between patients and controls was observed as Fas is constitutive. However, normal salivary glands displayed a faint signal of Fas receptor. Meanwhile, Sjögren's salivary glands exhibited a strong Fas signal. It seems that the glandular inflammation over-expresses the Fas receptor. In sharp contrast, the FasL was expressed exclusively in Sjögren's salivary glands, but not in controls. Moreover, the FasL was broadly distributed in inflammatory infiltrates, suggesting that the main source of FasL is lymphocyte-dependent (Figure 1 and Table I).

Caspase 3 is a crucial molecule for apoptosis; consequently it was important to define its presence in ductal epithelia and acini. Control biopsies displayed a faint signal of caspase 3; Sjögren's salivary glands expressed a strong signal of caspase 3 in ducts, acini and along inflammatory infiltrates. Because the detection of Fas/FasL pair and Caspase 3 does not necessarily implicate functional ac-

tivity, a complementary TUNEL assay was performed with the following results: Sjögren salivary glands showed positive TUNEL assays, apoptotic tags were distributed along ductal epithelial and acinar cells and were coincident with the Fas/FasL and Caspase 3 presence (Figure 2).

Proliferation markers: PCNA and Ki-67 proteins are considered reliable markers of proliferation. No proliferation markers were detected in control biopsies. PCNA was broadly expressed in Sjögren's tissues. Ki-67 was also detected in Sjögren's salivary glands, but the signal was weak. PCNA and Ki-67 were mainly detected along the inflammatory cell infiltrates and ductal epithelia and were absent in acinar cells (Figure 3).

Discussion

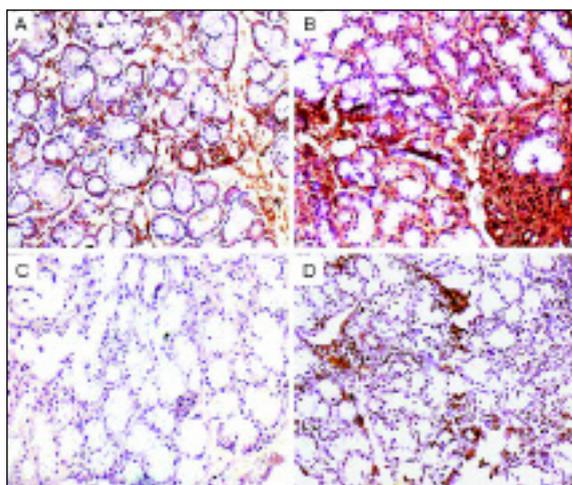The present study demonstrates that cells of the salivary glands from Sjögren's patients exhibit

Figure 1. Fas receptor distribution in salivary glands of controls (A) and Sjögren disease (B). FasL distribution in controls (C) and in Sjögren disease (D).
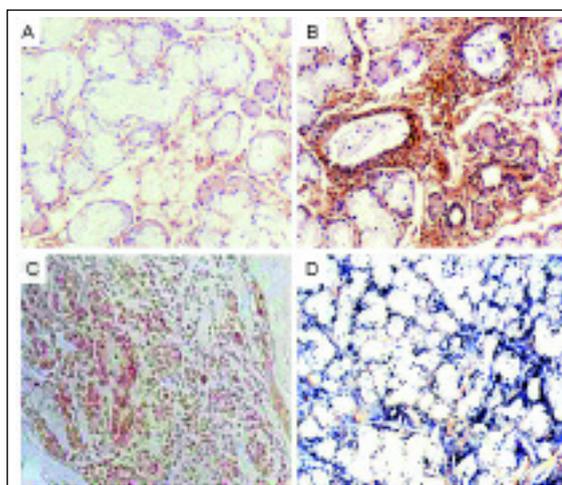

Figure 2. Caspase 3 distributions in salivary glands of controls (A) and Sjögren disease (B). TUNEL in negative controls (C) and positive in Sjögren salivary glands (D).

Table I. Apototic and proliferation markers in salivary glands

| Molecule	Controls	Distribution	Sjögren	Distribution
FasR	23/24	Faint in epithelial ducts	24/24	Broad, lymphoepithelial and acini
FasL	0/24	Absent	24/24	Broad, inflammatory cells
Caspase 3	0/24	Absent	17/24	Broad, lymphoepithelial lesions
TUNEL	1/24	Limited in ducts	24/24	Broad, lymphoepithelial and acini
PCNA	0/24	Faint in ductal epithelium	24/24	Broad, lymphoepithelial lesions
Ki-67	0/24	Absent	10/24	Faint, Lymphoepithelial lesions
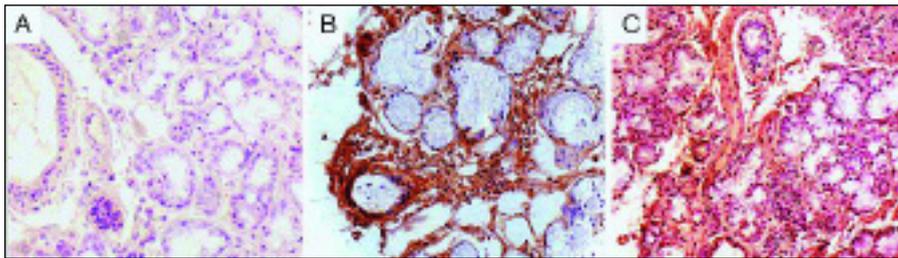
Figure 3. Proliferation markers. PCNA expression in salivary glands of controls (A) and in Sjögren disease (B). Ki-67 expression in Sjögren disease (C).

apoptotic features; inflammatory infiltrates in Sjögren's Syndrome display proliferation markers in lymphoid cells and this biological paradox inflammation-apoptosis in Sjögren's disease frequently results in atrophy and secretor dysfunction.

Among the possible mediators of apoptosis in Sjögren's syndrome are the CD8 cells, who may release proteases such as perforin or granzyme B.[7] Another route by which CD8 cell may induce apoptosis is by triggering the Fas pathway, because under stimulation FasL is expressed on its surface. According to our findings, the apoptotic targets are the epithelial and the acinar cells, rather than the infiltrating lymphocytes, which seem to be unable to cause autocrine or paracrine apoptosis. Simultaneous expression of Bcl-2 and FasL in CD8 lymphocytes may increase their resistance to apoptosis.[8]

The presence of CD4 cells in the inflammatory infiltrates of Sjögren's syndrome may be involved in apoptosis. In the animal model of Sjögren's syndrome, glandular apoptosis is induced via CD4 cells. During this process, a cleavage of the 120 kDa alpha-fodrin takes place. Interestingly, this autoantigen is important in triggering the disease in animal models and in humans. This effect is blocked by inhibitors of caspases.[9]

Another mechanism of apoptosis has been proposed in Sjögren's disease: anti-Ro and anti-La autoantibodies from Sjögren's patients may induce apoptosis of the A-253 cell line in a caspase-dependent manner, but this mechanism remains to be clarified.[10,11]

Cell proliferation in Sjögren's disease was recognized by Mickulicz in 1892, who reported a 42-year-old male farmer with lacrimal, parotid, and submandibular enlargement. The excised glands were filled with small round cells (lymphocytes). The patient died, possibly from a lymphoma, perhaps even Waldenstrom's macroglobulinemia.[12] Salivary glands in Sjögren's disease may develop benign lymphoepithelial lesions and ductal lesions, which are followed by basal cell hyperplasia and aberrant differentiation into a multi-layered and reticulated epithelium. All these changes are the result of cell proliferation. Morphological changes are accompanied by alteration in the cytokeratin pattern and enhanced Ki-67 protein expression.[13] Interestingly, a constant finding in target organs is a progressive focal infiltration of mononuclear lymphoid cells that replace the glandular epithelium. Different cytokines and chemokines are involved in B cell maturation and proliferation, the B cell activating factor being involved in this process.[14] In few cases of Sjögren's syndrome, the uncontrolled proliferation may result in malignant lymphoproliferative diseases.[15]

In summary, there is growing evidence that cytokines, chemokines and proliferation factors create a favourable glandular environment that alters the cell cycle resulting in proliferation of few epithelial areas and lymphoid foci.

Finally, the present investigation demonstrates that inflammatory cells play an important role in this paradox of the cell cycle, by which apoptosis and proliferation can co-exist in the same tissue target, leading to atrophy and loss of function.

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References
1. Fox RI, Stern M, Michelson P. Update in Sjögren syndrome. Curr Opin Rheumatol 2000;12:391-398
2. Hansen A, Lipsky PE, Dorner T. Immunopathogenesis of primary Sjögren's syndrome: implications for

disease management and therapy. Curr Opin Rheumatol 2005;17:558-565

3. Humphreys-Beher MG, Brayer J, Yamachika S, Peck AB, Jonsson R. An alternative perspective to the immune response in autoimmune exocrinopathy: induction of functional quiescence rather than destructive autoagression. Scand J Immunol 1999; 49:7-10

4. Badillo Almaraz I, Avalos-Díaz E, Villalobos-Hurtado R, Herrera-Esparza R. FasL and Bax genes are differentially expressed in acinar epithelium and inflammatory cells of primary Sjögren salivary glands. Minerva Med 2003;94:341-345

5. Vitali C, Bombardieri S, Jonsson R et al. European Study Group on Classification Criteria for Sjögren's Syndrome. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis 2002;61:554-558

6. Morbini P, Manzo A, Caporali R et al. Multilevel examination of minor salivary gland biopsy for Sjogren's syndrome significantly improves diagnostic performance of AECG classification criteria. Arthritis Res Ther 2005;7:343-348

7. Fujihara T, Fujita H, Tsubota K et al. Preferential localization of CD8+ alpha E beta 7+ T cells around acinar epithelial cells with apoptosis in patients with Sjögren's syndrome. J Immunol 1999;163:2226-2235

8. Manganelli P, Fietta P. Apoptosis and Sjögren syndrome. Semin Arthritis Rheum 2003; 33:49-65

9. Hayashi Y, Arakaki R, Ishimaru N. The role of caspase cascade on the development of primary Sjögren's syndrome. J Med Invest 2003;50:32-38

10. Sisto M, Lisi S, Castellana D et al. Autoantibodies from Sjögren's syndrome induce activation of both the intrinsic and extrinsic apoptotic pathways in human salivary gland cell line A-253. J Autoimmun 2006;27:38-49

11. Mitolo V. Autoantibodies from Sjögren's syndrome trigger apoptosis in salivary gland cell line. Ann N Y Acad Sci 2007;1108:418-425

12. Mikulicz-Radeck Jv: Über eine eigenartige symmetrische Erkrankung der Thränen- und Mundspeicheldrüsen. Beiträge zur Chirurgie. Festschrift gewidmet Theodor Billroth. Stuttgart 1892:610-630

13. Ihrler S, Zietz C, Sendelhofert A, Riederer A, Löhrs U. Lymphoepithelial duct lesions in Sjögren-type sialadenitis. Virchows Arch 1999;434:315-323

14. Jonsson R, Bolstad AI, Brokstad KA, Brun JG. Sjögren's syndrome a plethora of clinical and immunological phenotypes with a complex genetic background. Ann N Y Acad Sci 2007;1108:433-447

15. Grosbois B, Jego P, Leblay R. Gougerot-Sjögren syndrome and malignant lymphoproliferative syndromes. Rev Med Interne 1998;19:319-324

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