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Presence of fibronectin peptides in saliva of patients with Sjögren's Syndrome: A potential indicator of salivary gland destruction

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Abstract

Objective: The purpose of this preliminary study was to monitor the degree of destruction of salivary glands in Sjögren disease by the detection of fibronectin peptides in patients' saliva. Study design: The sample consisted of 10 subjects divided in 2 groups, one with Sjögren disease and a control group. Saliva samples were submitted to an inmunodetection analysis. In addition, non pathological salivary glands, obtained from 2 subjects who underwent minor oral surgery, were incubated with leukocyte homogenates and analysed to compare the obtained fragments. Results: The inmunodetection analysis of Sjögren saliva revealed multiple protein bands, including fibronectin, that were not present in saliva from healthy individuals. The inmunostained bands varied depending on the course of the disease, showing more fibronectin fragments in an active phase . Furthermore, results obtained from the non-pathological glands incubated with leukocyte homogenates were similar to those revealed in saliva from Sjögren patients. Conclusion: The presence of fibronectin peptides in Sjögren patients' saliva can constitute a method to monitor activity in Sjögren's disease.

Key words: *Sjögren's disease, salivary glands, saliva, fibronectin, leukocytes, elastase, collagenase, catepsin.*

Introduction

Sjögren´s syndrome is a chronic autoimmune disorder, characterized by lymphocytic infiltration and malfunction of exocrine glands. Lachrymal and salivary glands are most frequently involved, resulting in a drastic reduction of saliva and tear production (1-3). The primary form of Sjögren's disease (often called sicca syndrome) involves both keratoconjuntivitis, sicca and xerostomia (4). The syndrome can also appear associated to connective tissue diseases, usually rheumatoid arthritis (secondary Sjögren) (4).

The cellular mechanisms of Sjögren's disease are still not well known, but there are evidences indicating that an abnormal immune response against gland antigens could be in the origin of this pathology. In fact, the presence of infiltrating lymphocytes and inflammatory cells in glands and the production of auto-antibodies by activated B cells, strongly support the involvement of the immune system in the ontogeny of this disease (1, 4–9).

The diagnosis of Sjögren syndrome has been established last years with revised international criteria (Table 1). The diagnostic criteria is currently based on the ocular and oral symptoms, the objective evidencia of xeroftalmia (Schirmer test or Bengala rose test), salivary gland involvement in patients, and presence of autoantibodies to Ro/SSA or La/SSB antigens, or both, in the serum (10). However, the morphological analyses of salivary gland biopsy (presence of more than one focus of lymphocyte infiltrates containing >50 cells in a 4 mm2 square, together with evidences of parenchymal cell destruction) constitute the most sensitive and specific indicator for diagnosis and monitoring the activity of the disease (2,3,11,12).

In a research aimed to find biochemical markers of salivary gland destruction, we investigated the appearance of fibronectin in parotideal saliva of patients. Because of its large size (2x 220 KDa), this protein is a trace component of normal saliva (13), but antibodies directed to human fibronectin revealed the existence of many peptides in saliva of Sjögren patients. Based on the similarities among the observed fibronectin peptides in pathologic saliva and those from digests of fibronectin or healthy salivary glands with leukocyte proteinases, it appears that the peptides present in Sjögren's saliva are produced by leukocyte proteolytic enzymes, among them elastase. The results of our work provide evidences indicating that leukocyte proteolytic enzymes are involved in the destruction of salivary glands. This causal relationship gives scientific support to the measurement of fibronectin fragments in saliva as a non-invasive, potentially useful, method to monitor the extent of destruction of salivary glands in Sjögren's disease.

Table 1. Revised international criteria for the diagnosis of Sjögren´s syndrome.

I. Ocular symptoms	1) Have you had daily troublesome dry eyes for > 3 moths? 2) Do you have a recurrent sensation of sand o gravel in the eyes? 3) Do you used substitutes > 3 times a day?
II. Oral symptoms	1) Have you had a daily feeling of dry mouth for > 3 months? 2) Have you had recurrent or persistent swollen salivary glands as an adult? 3) Do you frequently drink liquids to aid in swallowing dry food?
III. Ocular involvement	1) Schirmer´s test (≤5 mm/5 min). 2) Rose Bengal.
IV. Salivary gland involvement	1) Unstimulated whole salivary flow (sialometry ≤ 1.5 ml/15 min). 2) Parotid sialography with the presence of difuse sialectasis and without evidence of obstruction in the major ducts. 3) Salivary scintigraphy showing delayed excretion o reduced concentration.
V. Minor salivary glands biopsy	Focal lymphocytic sialoadenitis with a focus score ≥ 1, defined as a number of lymphocytic foci (that are adjacent to normal-appearing mucous acini and contain > 50 lymphocytes/ 4 mm^2 of glandular tissue.
VI. Presence of autoanbodies to Ro/SSA or La/SSB Ags in the serum	
In patients without any potencially associated disease, primary Sögren´s syndrome can be defined as the presence of any four of the six diagnostic criteria above, as long as either item V (histopatology) or VI (Serology) is positive; or as the presence of any three of the four objective criteria items (items III, IV, V, VI). In patients with a potencially associated connective disease, the presence of item I or item II plus any two of items III, IV or V might be considered as indicative of secondary Sjögren´s syndrome. Exclusion criteria: Past head and neck radiation treatment, hepatitis C infection, AIDS, pre-existing lymphoma or sarcoidosis, graft versus host disease and use of anticholinergic drugs.	

Materials and Methods

Materials. Nitrocellulose sheets were obtained from Millipore (HAWP304F0). 3-amino-9-ethyl-carbazol was from Sigma (A-5754). Human leukocyte elastase (E.C.3.4.21.37), specific activity 50 U/mg (12), was also from Sigma (E-8140). Collagenase was from Boehringer (A 103578, lot 83697120). Fibronectin was obtained from human plasma by affinity chromatography on gelatine-sepharose as described (14,15). Peroxidase-labelled goat anti rabbit IgG was purchased from Dako (P0448). Antibodies against human fibronectin were prepared as previously described, and the specificity was properly tested (14,15).

Patients. Five adult individuals suffering Sjögren disease were chosen for this study. The diagnosis of the syndrome was based on diagnostic criteria. All patients were submitted to an hystopathological analysis of the minor labial salivary glands. Two subjects who underwent minor oral surgery (extirpation of a fibroma), were used as a source for non-pathologic salivary glands. Saliva from five individuals with no oral pathology was also used in the study as controls.

Clinical samples. Whole and parotidal saliva was obtained both from healthy and Sjögren (9) patients after extensive mouth washes by mechanical suction at different stages of the disease, whenever possible. The saliva samples were centrifuged (10 min x 10.000 rpm) to remove cells and cell debris and subsequently frozen at -40ºC until use. Minor labial salivary gland biopsies were obtained from patients in the course of minor surgery in the oral cavity after informed consent.

Preparation of leukocyte homogenates. Buffy-coats (200 ml) were obtained from pooled healthy human blood from the Central Blood Bank of Valencia. After centrifuging the cells through percoll, the upper cloudy band containing leukocytes (2-3 ml) was gently transferred to eppendorfs. Cells were washed with PBS by centrifugation (5 min x 3000 rpm), and diluted 1/10 in erythrocyte lysis buffer (0.83% NH4Cl, 0.1% HCO3Na, 0.04% EDTA). Finally, the tubes were centrifuged (5 min x 3000 rpm). The pellet was washed once with PBS by centrifugation (5 min x 3000 rpm) resuspended in 300µl PBS, three times frozen and thawed in liquid N2, and homogenised to break the cells. After centrifuging to remove cell debris (3 min x 15,000 rpm), the supernatant was stored at -70ºC until use.

Immunodetection of fibronectin fragments in saliva. Samples of saliva (20-30 µl) were diluted 1:1 v/v in 160 mM Tris-HCl buffer pH 6.8 containing 4% SDS and 3% dithiothreitol and run in a polyacrylamide gel (12.5%) under denaturing conditions. After finishing the run, half of the gel was stained for proteins either with coomasie brilliant blue or with silver nitrate (16), while the other half was electrophoretically transferred to nitro-cellulose sheets using a semi-dry blotting system (17,18). The electrophoretic blotting was carried out using a low-ionic strength buffer (3.7 mM Tris, 0.75 mM barbituric acid, 7.5 mM glycine, pH 8.9, containing 0.5% SDS and 0.1% mercaptoethanol), at 8 V/cm for 2-3 h. To visualise fibronectin and fibronectin fragments, the blot was stained by an indirect immunoperoxidase technique. Briefly, the nitro-cellulose sheet was first soaked for 15 min in PBS-tween (50 mM phosphate buffer pH 7.2, 0.9% NaCl, 0.05% tween-20) to remove mercaptoethanol and SDS, and then overnight incubated with PBS-tween containing 10% dry skimmed milk. After repeated washes, with PBS-tween, the sheet was incubated for 2h at 37ºC with rabbit immunoglobulins against human fibronectin (1:1000, final concentration in PBS-tween, 3% skimmed milk), and then thoroughly washed with PBS-Tween. The second antibody was peroxidase-labelled goat anti-rabbit IgG and was diluted 1:2000 in PBS-tween 3% skimmed milk. The blot was incubated for 1 h at 37ºC and after, repeated washes, further incubated at 37ºC with the chromogenic solution (0.05% 3-amino-9-ethyl carbazol, 0.001% H2O2 in 50mM citrate buffer pH 5). Coloured bands appeared within 1 h (18).

Proteolytic digestion of fibronectin and salivary glands. A sample of fibronectin (15 µg) in PBS was incubated with 2 µl of human elastase (2 mU), collagenase (0.3 mU) or leukocyte homogenate for 15 min at 37ºC. The tubes were centrifuged and an aliquot of the supernatant was analysed by gel electrophoresis and immunodetection, as described above. In parallel, a biopsy of labial salivary gland was gentle homogenized in 50 µl of PBS, 3x washed by centrifugation to remove contaminating plasma proteins, and 30 µl of the suspension was incubated with 2 µl of leukocyte homogenate for 15 min at 37ºC. After centrifugation to remove cell debris (5 min x 15.000 rpm), an aliquot of the supernatant was analysed by gel electrophoresis, transferred to nitrocellulose and stained with antibodies to visualize the presence of fibronectin fragments, as described above.

Results

Presence of fibronectin fragments in saliva of Sjögren patients. Samples of saliva from Sjögren patients were analysed by electrophoresis in 12.5% polyacrylamide gels under denaturing conditions. After protein staining, several bands were observed evidencing the presence of significant amounts of protein in the saliva of patients (data not shown). On the contrary, the presence of protein bands in control saliva could only be visualised after protein silver staining. A replicate of this gel containing the same samples was electrophoretically transferred to nitrocellulose sheets and incubated with specific antibodies anti human fibronectin. As shown in (Fig.1,2), multiple protein bands became noticeable in pathologic saliva, revealing the presence immunologi-

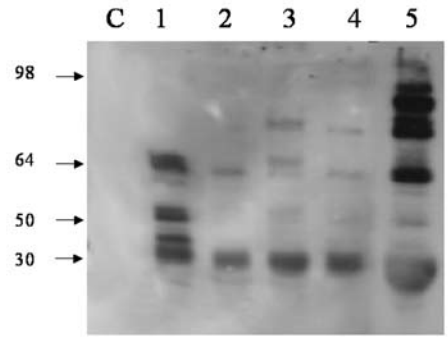

Fig. 1. Presence of fibronectin fragments in saliva of Sjögren patients. Samples of saliva (control and patients 1 to 5) were electrophoretically separa-ted in polyacrylamide gels, transferred to nitrocellulose sheets and immunodetected by means of rabbit IgG anti-human fibronectin and peroxidase-labelled goat anti rabbit IgG. A chemilluminescent substrate (ECLTM) was used to visualise the fibronectin immunoreactive bands.

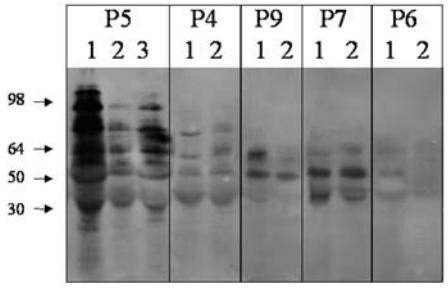

Fig. 2. Changes in fibronectin peptides in saliva of Sjögren patients. Samples of saliva from patients Nr. 4 (P4), 5 (P5), 6 (P6), 7 (P7) and 9 (P9), taken at different times in the course of the disease. Patient's 5 samples were taken at 15 (lane 2) and 24 months (lane 3); all the others taken 24 months after the first sample (lanes 2), electrophoretically separated in polyacrylamide gels, transferred to nitrocellulose sheets and immunodetected with anti-human fibronectin IgG and peroxidase-labelled goat anti rabbit IgG. The use of a chemilluminescent oxidation substrate allowed the visualization of several immunoreactive bands.

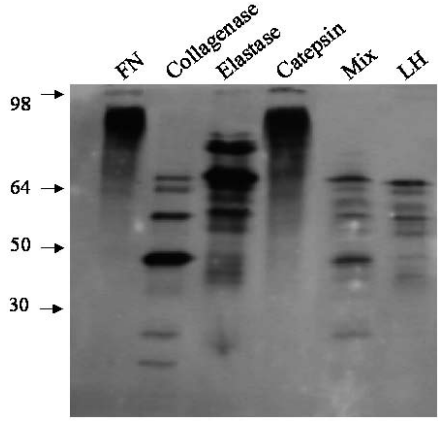

Fig. 3. Proteolytic digestion of fibronectin by leukocyte homogenates. A sample of pure fibronectin (FN) was incubated either with purified human leukocyte collagenase (Co), human leukocyte elastase (E), catepsin (Ca), mixed collagenase, elastase and catepsin (Mix) or crude human leukocyte homogenate (LH), and analysed by gel electrophoresis under denaturing conditions. The gel was transferred to nitrocellulose sheets and immu-nodetected (western blot) with antibodies anti-human fibronectin and peroxidase-labelled second antibody.

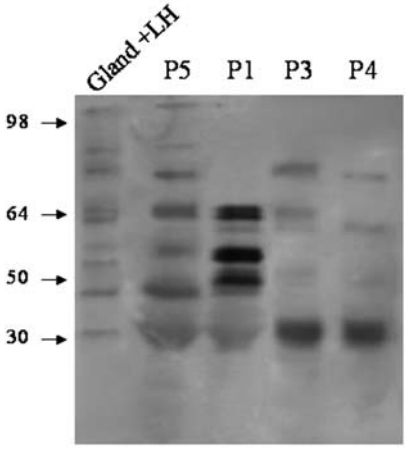

Fig. 4. Release of fibronectin peptides from extracellular matrix of salivary glands by leukocyte proteinases. A minor salivary gland from healthy was incubated for 2 h at 37ºC with a crude leukocyte homogenate, was withdrawn, centrifuged, and the supernatant submitted to gel electrophoresis(G+LH). Samples of saliva from patient 5 (P5), 1 (P1), 3 (P3) and patient 4 (P4) were loaded in the polyacrylamide gel and blotting to nitrocellulose sheets. The presence of fibronectin peptides was evidenced by immunodetection with anti fibronectin antibodies.

cally-related fibronectin peptides in saliva of patients (lanes 1-5). In saliva from a representative healthy control (C), these bands were absent.

The immunostained bands varied in the course of the disease. Thus, for patient 5, a sample taken during an active phase of the disease (I) showed more fibronectin fragments than in a silent phase (P5-II ; P9-II ; P6-II). Reactivation of clinical symptoms resulted in increased of immunoreactive fibronectin fragments in saliva (P5-III).

Proteolytic digestion of fibronectin by leukocyte homogenates and leukocyte proteolytic enzymes. A sample of soluble fibronectin (97% homology to cellular fibronectin) was incubated either with collagenase, elastase, catepsin, the three enzymes (Mix) or human leukocyte homogenate from a buffy coat (LH). The incubated sample was analysed by electrophoresis in polyacrylamide gels and immunostained with antibodies anti-fibronectin (Fig.3), shows the tryptic map obtained upon incubating fibronectin with the various proteolytic enzymes. While collagenase, as well as elastase efficiently digested fibronectin, catepsin was not active. The fragments obtained after incubating fibronectin with a crude leukocyte homogenate (LH) were very similar to those found after incubating the protein with the mixture of the three enzymes.

Involvement of leukocyte prote olytic enzymes in the destruction of salivary glands. To mimic the situation that might occur in the course of Sjögren's disease, minor salivary glands from healthy individuals were homogenized, washed with PBS to remove contaminating plasma proteins, and incubated for 2 h at 37ºC with a crude leukocyte homogenate. Aliquots of this sample, together with saliva of Sjögren patients (P1-P5) were analysed by electrophoresis (SDS-PAGE), transferred to nitrocellulose sheets and stained with anti-fibronectin antibodies. The results displayed in (Fig.4), showed the presence of soluble fibronectin fragments in the incubated homogenates of salivary glands similar in size to those appearing in saliva of Sjögren patients.

Discussion

Saliva contains several proteins; some of them are produced by the exocrine gland (i.e. lysozyme) while others are of plasma origin (19). The tight junctions within acinar cells and the reduced permeability of the cellular membrane towards plasma proteins represent a barrier preventing the bulk of plasma proteins to escape (20). However, traces of plasma proteins can be detected in normal saliva up to molecular weights of 60-70 KDa (21).

Fibronectin is a gene product that by alternative splicing originates a 2x220 KDa heterodimer that is found circulating in plasma, and a cellular insoluble isoform associated to the extracellular matrix of many tissues, among them, exocrine glands (i.e. salivary glands) (22,23). This so-called "cellular fibronectin" is thought to mediate cell-cell and cell-biomatrix attachment through specific receptors and adhesins and to play an important role in cell migration and organ differentiation (24). Because of its large size, plasma fibronectin cannot cross the barrier represented by acinar cells, while cellular fibronectin is insoluble. Consequently fibronectin is a trace protein in normal saliva (13,25). Based on these facts, we hypothesized that the appearance of fibronectin in saliva could only be possible: a) if the permeability of the cellular barrier to plasma proteins had been altered after acinar tissue damage, or b) if the cellular fibronectin, located mainly at the extracellular matrix of ductal cells, had been released by hydrolytic cleavage (26).

The analysis of saliva from healthy individuals with specific anti-fibronectin antibodies by immunoblotting revealed the total absence of the 220 KDa fibronectin band, in agreement with previous findings (27). On the contrary, in saliva of patients with Sjögren's syndrome, several immunoreactive fibronectin peptides, but not whole-size fibronectin, was noticeable (Fig.1). This excludes the plasma origin of most (if not all) of the immunoreactive fibronectin found in pathological saliva.

The amount of fibronectin fragments present in pathological saliva seems to be associated with the severity of the disease. Thus, in the patients who reported themselves at different stages of their disease, significant changes in saliva could be observed, as illustrated in (Fig.2).

A common finding in Sjögren's syndrome is the presence of leukocyte infiltrate in the glands (5,6,9). In an attempt to establish a link between the presence of leukocytes and the destruction of the tissue, fibronectin was incubated with leukocyte proteinases (i.e. collagenase, elastase and catepsin), as well with crude leukocyte homogenate. The experiment shown in (Fig.3) evidenced the major role played by collagenase and elastase in the proteolytic fragmentation of fibronectin by leukocytes.

The immunoblotting of peptides obtained after digestion of salivary glands with leukocyte homogenate provided a further piece of evidence to support our hypothesis. The proteolytic enzymes present in leukocyte homogenates were able to digest the, otherwise insoluble, cellular fibronectin. Moreover, the similarities among the tryptic digests (Fig.4), suggest that the proteolytic activities of infiltrating leukocytes may be directly involved in the destruction of the salivary gland. As a result of their action, soluble small peptides would be released from the extracellular matrix and appear in saliva of Sjögren patients.

The rate of saliva production is currently used as diagnostic parameter in Sjögren's syndrome (28,29), as well as other diagnostic techniques (30-32), however mor-

phological examination of salivary gland biopsy is still the most conclusive parameter to monitor the course of irreversible gland destruction (3). However, because of the discomfort caused to the patients, this invasive test cannot be used for regular examination of the activity of the disease.

Attempts have been made to monitor in patient's saliva proteins that could be indicative of an active inflammatory process (i.e. calprotectin; 28); output of plasma small-sized proteins like beta 2-microglobulin. However, despite of initial promising results, the validity of such parameters remain controversial (27,33-35).

The presence of fibronectin proteolytic fragments in patient's saliva generated by leukocyte proteinases, as shown in this paper, could constitute the basis for a non-invasive approach to monitor the activity of Sjögren disease. To verify the clinical usefulness of this test, further research is in progress.

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