Specific Proteins Identified in Whole Saliva from Patients with Diffuse Systemic Sclerosis

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ABSTRACT. ***Objective.*** To evaluate the global changes of salivary protein profiles in patients with systemic sclerosis (SSc) using a proteomic approach.

Methods. Whole saliva (WS) was collected from 15 patients with diffuse SSc and 15 healthy volunteers. Protein expression profiles for each sample were generated by 2-dimensional gel electrophoresis, and protein spots of interest were identified using peptide mass fingerprinting.

Results. The level of all the most representative salivary proteins except keratin 6L remained unchanged and only qualitative differences were observed between control subjects and patients with SSc. A total of 19 spots were found in SSc that were not matched with the controls. Fourteen out of a total of 19 spots were identified by mass analysis and were found to collapse into 9 unique proteins. These spots were identified to be cyclophilin A, calgranulin B, psoriasin, $ß_2$-microglobulin, calgranulin A, glyceraldehyde-3-phosphate dehydrogenase, triose phosphate isomerase (TPI), actin-related protein 2/3 complex subunit 2 (Arp2/3 complex), and cystatin B.

Conclusion. Our study is the first reporting the WS protein pattern of patients with SSc and comparing the differences between WS of patients with SSc and WS of healthy subjects. Both previously identified and newly identified proteins were detected in WS using a proteomic approach. Some of these proteins, like keratin 6L, psoriasin, TPI, and Arp2/3 complex, might have a pathological significance for SSc. It is possible that some of them can be defined as new therapeutic targets or diagnostic markers for SSc disease. (First Release August 15 2007; J Rheumatol 2007;34:2063–9)

Key Indexing Terms:
SALIVA SYSTEMIC SCLEROSIS PROTEOMICS

Systemic sclerosis (SSc) is a generalized connective tissue disorder, characterized by a wide spectrum of microvascular and immunological abnormalities, leading to progressive accumulation of extracellular matrix components in the skin and visceral organs[1]. The disease can involve every organ and system such as the lungs, gastrointestinal tract, heart, and kidney, and it has been shown that salivary glands can also be subjected to fibrosis in patients with SSc[2-4]. The progressive fibrosis is a consequence of multiple events that lead to increased biosynthesis of matrix proteins by connective tissue cells. Apoptosis of endothelial cells, fibroblast dysregulation, and aberrant recruitment of inflammatory cells into the perivascular dermal matrix of the skin and internal organs are among the most widely recognized elements involved in the pathogenesis of SSc[5-14]. In view of the complex mechanisms of SSc and its broad clinical spectrum, we used a proteomic approach to research the presence of proteins in salivary fluid that might be potential biomarkers of the disease. In recent years, interest has arisen in application of proteomic analysis to rheumatic diseases (i.e., Sjögren's syndrome, osteoarthritis)[15-17]. Nonetheless, at present, in SSc the proteome analysis approach has been restricted to the study of protein bronchoalveolar lavage fluid profiles[18]. In this study, for the first time, we used 2-dimensional gel electrophoresis (2-DE) in combination with mass spectrometry to obtain a protein map of whole saliva (WS) of patients with SSc, and compared it with WS from healthy subjects in order to evaluate the global changes of the protein profiles that occur in this disease.

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MATERIALS AND METHODS

Chemicals. Iodoacetamide (IAA), dithiothreitol (DTT), 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS), urea, thiourea, glycerol, and 30% acrylamide-N,N,N bisacrylamide were acquired from Sigma-Aldrich Co. (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS), ammonium persulfate (APS), N,N,N',N'-tetramethylenediamine (TEMED), and glycine were purchased from MP Biomedical, LLC (Eschwege, Germany). IPGs pH 3-10 L, pharmalyte 3-10, and dry strip cover fluid were

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purchased from Amersham Biosciences (Uppsala, Sweden). Coomassie Brilliant Blue G 250 was from Merck (Darmstadt, Germany). All other reagents were from standard commercial sources and were of the highest grade available.

Human subjects. Fifteen women with a diagnosis of diffuse SSc (mean age 50.5 ± SD 12.2 yrs) according to the International Classification Criteria for the disease[19] and 15 healthy volunteer women with similar mean age (47.3 ± 16.2 yrs) and demographic characteristics were enrolled. Informed consent was obtained for diagnostic or clinical purposes. At the time of sample collection, all the patients showed moderate disease activity, which varied between 2.5 and 4[20]. The disease pattern was suggestive of a diffuse skin involvement (> 14 extent of skin involvement) according to modified Rodnan skin score, not associated with active acral ulcers and severe gastroesophageal reflux disease with esophagitis. All the patients presented similar clinical features with a reduction of DLCO (mild in 11/15 and moderate in 4/15) and bilateral basal lung interstitial disease at the HRCT. Polyarthralgias were documented in 6 out of 15 patients and 5 patients presented melanodermia. No patient was affected by a secondary Sjögren's syndrome. At the time of sample collection patients were taking calcium antagonist, corticosteroids, or nonsteroidal antiinflammatory drugs. The serological profile was positive for Scl-70 autoantibodies. Moreover, no subject presented major dental disorders and salivary gland damage. Persons who smoked were excluded from the study. Mean time between onset of the disease and the time of sample collection was 3 years (range 2–4 yrs) and therefore patients presented with early diffuse SSc.

Sample collection and preparation. WS samples were collected early in the morning in standard conditions, i.e., all subjects were asked to have an empty stomach, without having consumed any food or drink (including gum or candies) since the night before. Sample preparation was performed as described[15]. In particular, in order to minimize degradation of the proteins, the samples were processed immediately and kept on ice during the process. Between 1 and 2.5 ml saliva was obtained from each subject. Salivary pH (mean ± SD) was recorded in patients with SSc (pH 7.03 ± 0.38) and controls (pH 7.00 ± 0.46). To remove debris and cells, centrifugation at 14,000 g was performed for 30 min at 4°C. Proteins from resulting supernatants were precipitated using 10% (w/v) trichloroacetic acid and 0.05% DTT. After incubation at 0°C for 1 h, insoluble material was pelleted at 14,000 g. Pellets were washed 3 times with pure acetone, air dried, and solubilized in 7 M urea, 2 M thiourea, 4% CHAPS, 60 mM DTT, 0.5% 3–10 ampholytes, and 0.002% of bromophenol blue (rehydration solution). The protein amount was estimated using an RC DC protein assay from Bio-Rad. This colorimetric assay allows the determination of protein concentration in the presence of reducing agents and detergents. Bovine serum albumin was used as a standard. Protein loads were 150 µg for analytical and 1500 µg for preparative gels.

2-DE analysis. 2-DE was performed using the Immobiline-polyacrylamide system as described[15]. Samples were applied by in-gel rehydration for 10 h using low voltage (30 V) in pH 3–10L, 18-cm IPG strips. The proteins were then focused for up to 70,000 Vh at a maximum voltage of 8000 V. For preparative run a preliminary step at 200 V for 12 h was introduced. The second dimension (sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SDS-PAGE) was carried out by transferring the proteins to 12% polyacrylamide gel (18 cm × 20 cm × 1.5 mm) running at 40 mA/gel and at 10°C for about 6 h.

Staining and image analysis. Analytical gels were stained with ammoniac silver nitrate as described[21]. Preparative gels for mass spectrometric analysis were stained with Coomassie Brilliant Blue G-colloidal according to Candiano, *et al*[22]. The stained gels were scanned with an Epson Expression 1680 Pro scanner and the images were analyzed with Image-Master 2D Platinum 5.0 (Amersham Biosciences) software program. Both analytical and preparative gels showed the same protein pattern. Spots were automatically detected, manually edited, and then counted. After spot detection in gels, a match set and a synthetic image for each class was generated. The synthetic gels were obtained by averaging the positions, shapes, and optical densities of the matched spots in a given set of gels of the same class. This produces an intersection of all the gels, showing only the spots found in all (100%) the given images for each class. Nonetheless, each variation was controlled with sample by sample inspection to prevent true quantitative variations appearing as qualitative ones. Regarding quantitative analysis, the percentage of the volume of the spots representing a certain protein was determined in comparison with the total proteins present in the 2-D gel. The 2-D electrophoresis profiles and relative spot intensities obtained for most of the samples were perfectly reproducible when performed in duplicate or triplicate.

Statistical analysis. All values were expressed as mean values ± SEM. Statistical analysis of Image-Master results by t-test and then Microsoft Office Excel software allowed the study of proteins that were significantly increased or decreased in SSc with respect to control. A p value < 0.05 was considered significant. The proteins that showed statistically significant difference of intensity of expression (> 2-fold) were selected and identified.

In-gel digestion. Fragments of the gel containing proteins of interest were cut out for digestion with trypsin using the following procedure. Gel pieces obtained after 2-DE were treated with 50 mM ammonium bicarbonate and 30% acetonitrile (AcN) for 30 min at room temperature because disulfide bond-containing proteins were already reduced and alkylated after isoelectric focusing. Gel pieces were then dried for 30 min in a Hetovac vacuum centrifuge (Heto, Allerod, Denmark). Dried pieces of gel were rehydrated for 45 min at 4°C in 5–20 µl of a solution of 50 mM ammonium bicarbonate containing trypsin at 6.25 ng/µl. After overnight incubation at 37°C, the gel pieces were dried in a high-vacuum centrifuge before being rehydrated by the addition of 20 µl H_2O and finally dried again. Elution of the peptides was performed with 20 µl of 0.1% trifluoroacetic acid (TFA) for 20 min at room temperature with occasional shaking. The TFA solution containing the proteins was transferred to a polypropylene tube. A second elution of the peptides was performed with 20 µl of 0.1% TFA in 50% AcN for 20 min at room temperature with occasional shaking. The second TFA solution was pooled with the first one. The volume of the pooled extracts was reduced to 1–2 µl by evaporation under vacuum. 20 µl of 1% TFA was added and the volume reduced to 1–2 µl by evaporation under vacuum. Control extractions (blanks) were performed using pieces of gel devoid of proteins.

Protein identification by peptide mass fingerprinting. Before peptide mass fingerprinting, the volumes of peptide-containing solutions were adjusted to 5 µl by the addition of 0.1% TFA in 50% AcN. One microliter of each sample was deposited on a 2 × 96-well matrix-assisted laser desorption ionization (MALDI) target plate and dried in a vacuum container. Equal volumes of matrix (10 mg/ml a-cyano-4-hydroxycinnamic acid in 50% AcN, 0.1% TFA) were added to the previously loaded digest. Samples were dried using a vacuum container. Mass spectroscopy measurements were conducted with a MALDI/time of flight 2-D gel electrophoresis Voyager super STR (Applied Biosystems, Foster City, CA, USA) equipped with a 337-nm nitrogen laser. The analyses were performed in the reflectron mode with an accelerating voltage of 20 kV, a delayed extraction parameter of 350 ns, and a low mass gate of 850 Da. Laser power was set slightly above threshold (10–15% higher than the threshold) for molecular ion production. Spectra were obtained by the summation of 150–300 consecutive laser shots. The spectra were internally calibrated using lysozyme [LYSC_CHICK (P00698)] and externally calibrated using trypsin [TRYP_PIG (P00761)]. Masses of the peaks were extracted from the spectra and used for protein identification using the MASCOT 2.1 peptide mass fingerprint tool and ProFound search engine from PROWL (The Rockefeller University, New York, NY, USA; http://prowl.rockefeller.edu). The probability scores shown were those obtained by means of Mascot. The research was conducted against SWISS-PROT and TrEMBL databases (Release 49.0, February 7, 2006, contains 207'132 sequence entries, comprising 75'438'310 amino acids abstracted from 139'151 references). Restrictions were placed on species (human), mass tolerance (± 50 ppm), maximum missed cleavages by trypsin (up to 1), and cysteine modification by carbamidomethylation.

RESULTS

WS protein map of controls and patients with SSc. The protein composition of WS of healthy subjects and patients with SSc

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was analyzed by 2-D gel electrophoresis. Typical 2-D gel images of human salivary proteins, obtained with the broad-range pH strips (pH 3–10L) from control and disease samples, are shown in Figure 1. Following computational analysis, we detected about 200 protein spots on each gel using ammoniac-silver staining. Of the spots picked from the 2-D gels and submitted to peptide mass fingerprint analysis, 37 spots were identified (Figure 1) and collapsed into 24 separate proteins.

Identification of differentially expressed proteins. We analyzed the differences in the WS protein pattern, comparing the synthetic gels of the disease samples with respect to the controls (Figure 1). A good overlapping of representative salivary protein groups, for instance α-amylase (spot no. 40), albumin (spots 50, 51), actin (spots 35, 36), carbonate anhydrase VI

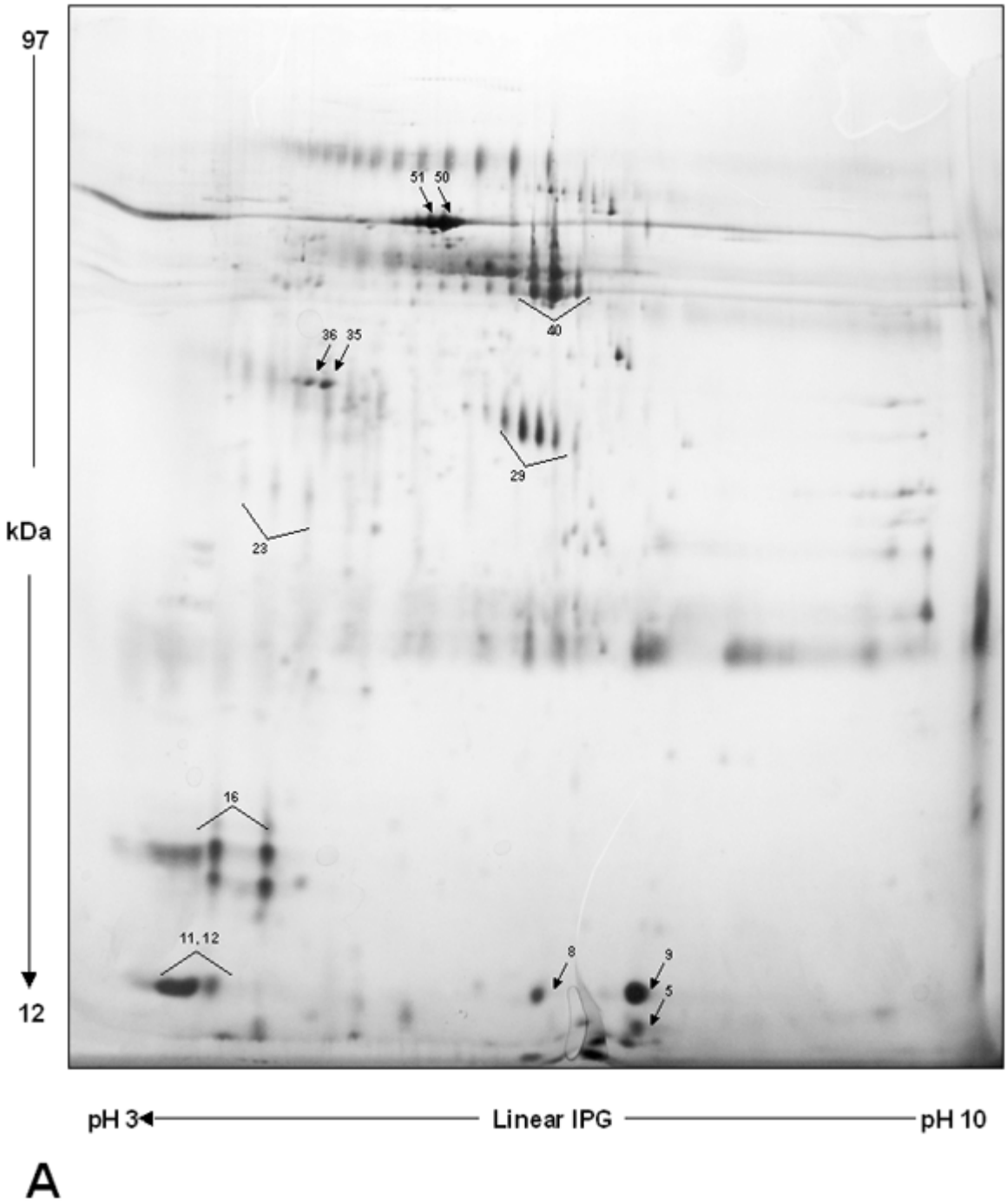

Figure 1. Representative 2-DE gel map of normal and SSc whole saliva proteins; 150 µg proteins were separated by 2-DE using 18 cm pH 3–10 linear strip and 12% SDS-PAGE. Proteins were detected by silver staining. The map was analyzed with Image-Master 2D Platinum Software. A. 2-DE gel map from normal saliva. B. 2-DE gel map from SSc saliva.

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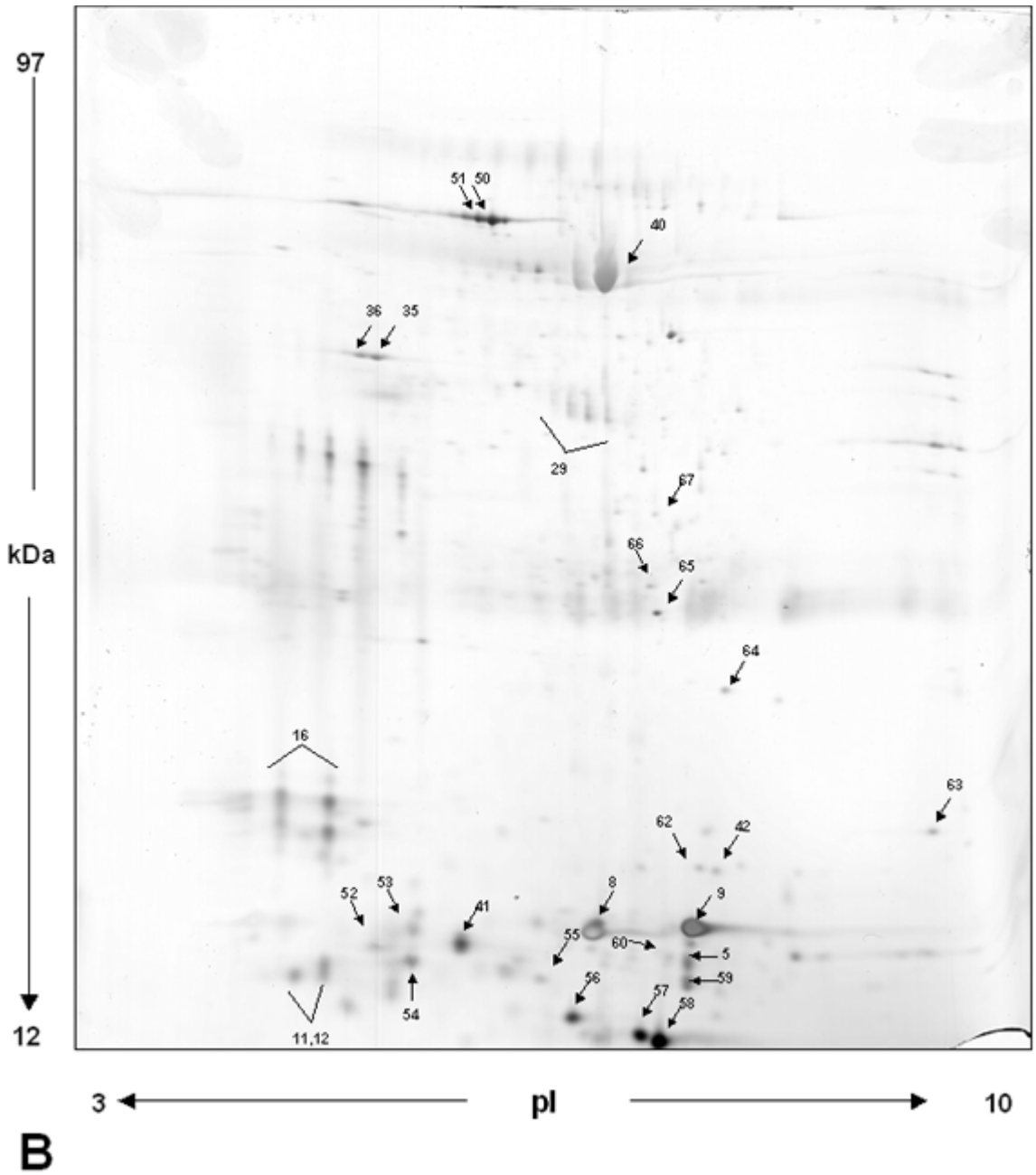

Figure 1. Continued

(spot 29), keratin 6L (spot 23), various cystatins (spots 5, 8, 9, 11, 12), and prolactin-inducible protein precursor (spot 16), was observed (Figure 1), and their identification has been described in healthy subjects and patients with pSS[15]. No significant quantitative differences emerged in the statistical analysis for these proteins except for keratin 6L, which was found to be upregulated 2.7-fold in the patients with SSc. Nevertheless, qualitative differences between the 2 groups were observed from the comparison of respective synthetic gels. Indeed, 19 spots were found in SSc that did not match with the controls. Fourteen out of a total of 19 spots were identified by mass spectroscopy analysis and were found to collapse into 9 unique proteins, while the remaining 4 spots (60, 62, 63, 66) were not identified because of their relative low concentration. Table 1 shows the protein names of the 10 spots identified in SSc gel that did not match with the controls. These spots were identified to be cyclophilin A (spot 42), calgranulin B (spots 41, 52, 53, 54), psoriasin (spot 55), $ß_2$-microglobulin (spot 56), calgranulin A (spots 57, 58), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (spot 64), triose phosphate isomerase (TPI; spot 65), Arp2/3 complex (spot 67), and cystatin B (spot 59). Other members of the cys-

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Table 1. Identification of 9 proteins that are present in whole saliva of only patients with SSc.

Spot	Protein	Swiss Protein Accession No.	Mr ($\times 10^3$)	pI	Matched Peptides	Score	Sequence Coverage, %	Function
41	Calgranulin B	P06702	13.2	5.71	9	65	76	Expressed by macrophages in acute and in chronic
52	Calgranulin B	P06702	13.2	5.71	7	71	55	inflammation. Seem to be an inhibitor of protein kinases.
53	Calgranulin B	P06702	13.2	5.71	7	60	70	Also expressed in epithelial cells constitutively or induced
54	Calgranulin B	P06702	13.2	5.71	5	39	53	during dermatoses. May interact with components of the intermediate filaments in monocytes and epithelial cells
55	Psoriasin	P31151	11.4	6.26	7	56	49	Fetal ear, skin, and tongue and human cell lines. Highly upregulated in psoriatic epidermis. Also highly expressed in the urine of bladder squamous cell carcinoma-bearing patients
56	$ß_2$-microglobin	Q6IAT8	13.8	6.06	4	38	37	The $ß_2$-microglobin is part of the MHC Class I molecule
57	Calgranulin A	P05109	10.8	6.51	11	97	63	Expressed by macrophages in chronic inflammation and in epithelial cells constitutively or induced during dermatoses
58	Calgranulin A	P05109	10.8	6.51	8	72	62	May interact with components of the intermediate filaments in monocytes and epithelial cells
59	Cystatin B	P04080	11.1	6.96	6	69	64	An intracellular thiol proteinase inhibitor. Tightly binding reversible inhibitor of cathepsins L, H, and B
42	Cyclophilin A	P62937	18	7.82	11	73	54	Peptidyl-prolylases accelerate the folding of proteins. It catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides
64	Glyceraldeyde-3 P-dehydrogenase	P04406	35.9	8.58	5	32	23	Carbohydrate degradation, glycolysis
65	Triose phosphate isomerase	P60174	26.8	6.51	15	125	62	Carbohydrate biosynthesis, gluconeogenesis
67	Actin-related protein 2/3 complex subunit 2	O15144	34.4	6.84	18	129	43	Part of a complex implicated in the control of actin polymerization in cells

tatins family were found to be expressed in the same way, both in SSc and in controls. Quantitative analysis revealed similar levels of spot intensities corresponding to cystatin-S (spots 11, 12), -SN (spots 8,9), and -D (spot 5) between SSc and controls.

DISCUSSION

We describe for the first time the human WS protein pattern of patients with SSc. Comparing the synthetic gels of SSc patients and healthy controls we found many differences that widely concern both protein-related inflammation and immune response and proteins related to tissue damage. At the same time, we found no quantitative difference in the expression of most of the so-called typical salivary proteins, such as α-amylase, prolactin-inducible protein precursor, albumin, or cystatins, alterations that we described in Sjögren's syndrome[15]. Among the proteins related to inflammation we specifically identified 3 proteins that belong to the S100 calcium- and zinc-binding protein family: calgranulin A or S100A8 (2 spots), calgranulin B or S100A9 (4 spots), and psoriasin or S100A7 (1 spot). The calgranulin A and B are mostly present in the cytosol of neutrophils[23]: these proteins are released from neutrophils as a heterotrimeric complex (S100A8/A9) under inflammatory conditions. It has been reported that amounts of S100A8/A9 in blood or extracellular body fluid are increased under many pathological conditions, for instance rheumatoid arthritis[24], inflammatory bowel diseases[25], tumors[26], and many inflammatory conditions[27]. The physiological role of this factor is not fully understood: proposed extracellular activities of the protein complex include antimicrobial, chemotactic activity against neutrophils and apoptosis-inducing activity in various cells by a mechanism of extracellular zinc exclusion[28]. Interestingly, a new finding[29] suggests that high concentrations of S100A8/A9 can inhibit the matrix metalloproteinases (MMP), a family of zinc-dependent enzymes, by the sequestration of zinc. A reduced activity or expression of MMP is an important feature of SSc and play a role to reduce matrix degradation and to determine extensive fibrosis of this disease[30]. In this context we suggest that the high concentrations of S100A8/A9 might play a role in inhibiting MMP activity.

Psoriasin was first identified in psoriatic skin[31], where it is upregulated. Psoriasin is thought to be involved in inflammation since it is a potent chemotactic agent able to stimulate the neutrophil and CD4+ lymphocyte infiltration in the psoriatic epidermis[31]. In addition, a stronger expression of psoriasin was also reported both in preinvasive and invasive malignant lesions of skin such as squamous cell carcinomas of the bladder[32]. In this tumor, a high level of psoriasin was found in the urine of patients, suggesting a putative role of urinary marker for this protein[32]. While different studies reported the presence of calgranulin A and B in the protein map of WS from healthy subjects[18], only a recent article[33] reported the presence of psoriasin in WS using the capillary isoelectric focusing-based multidimensional separation platform coupled with ESI-tandem mass spectrometry. This technique has a high

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resolving power with an enrichment of low-abundance proteins/peptides. Thus we might suppose that the quantity of psoriasin, when detectable, is very low in control WS. Our data suggest a strong expression of psoriasin only in patients with SSc. Although the biological effect of psoriasin in SSc is currently unknown, it is interesting to speculate that psoriasin may be important in the invasive phenotype. This role might be mediated through an indirect influence on the effector cells of the host immune response, or perhaps through a more direct influence on the stress oxidative response. The increase of psoriasin expression could be a consequence of the ischemic conditions that characterize SSc.

In addition to the S100 binding proteins, a group of other proteins were also found in SSc WS but not in the controls. The first was the Arp2/3 complex, which had never been identified previously in WS. The Arp2/3 complex regulates actin polymerization and cross-linking, and this complex is necessary for neutrophil chemotaxis and phagocytosis[34]. Another protein was the $ß_2$-microglobulin, which may reflect the immune dysregulation of SSc.

The presence of TPI and GAPDH in WS of SSc patients and not in controls is also intriguing. These glycolytic enzymes are normally sequestered in the cytoplasm and they are only released into the circulation in minute amounts during pathological states that correlate with cell damage or apoptosis. Among the clinical features of SSc there is vascular damage with severe ischemia[30]. Since the anaerobic condition needs greater amounts of glucoses than the aerobic condition to produce the same level of energy, the glycolytic enzymes would be of critical importance in the vascular cells. In this context, conditions of stress for the skin and vascular bed, such as those happening in SSc, may increase the need for TPI and GAPDH. Another possible role hypothesized for TPI and GAPDH is that each can act as an autoantigen in SSc as well as in other autoimmune disorders (i.e., chronic arthritis and systemic lupus erythematosus)[35,36].

Out of the 9 proteins not matching with controls, moreover, we could identify the proinflammatory cyclophilin A. Cyclophilin A is the prototype of a family of conserved proteins involved in many basic biological processes such as expression, folding, and degradation of proteins[37]. Interest has arisen in a hypothetical role of cyclophilin A in the pathogenesis of immune-mediated disorders[38] and evidence suggests that cyclophilin A may act as a novel biphasic paracrine and autocrine stimulatory factor for endothelial cells, contributing to the pathogenesis of immune-mediated endothelial activation and dysfunction[37].

Finally, concerning keratin 6L, the increase of this protein could be associated with the fibrosis process that characterizes the disease.

Our preliminary cross-sectional study, even with some limitations, has shown that the salivary protein pattern of SSc seems to be characterized by the expression of a mixture of inflammatory and immune-related proteins as well as by proteins correlated to oxidative stress damage. Although further investigation is required to explain the pathological significance of these alterations (i.e., repeated followup analysis, comparison with limited SSc and late diffuse SSc, parallel salivary biopsy histology), 2-DE in combination with mass spectrometry analysis appears to be a powerful method to identify new potential therapeutic targets or diagnostic markers for SSc disease.

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