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Autoantibodies from Sjögren's syndrome induce activation of both the intrinsic and extrinsic apoptotic pathways in human salivary gland cell line A-253

M. Sisto [a,*,1], S. Lisi [a,1], D. Castellana [a], P. Scagliusi [b], M. D'Amore [b], S. Caprio [a], A. Scagliusi [c], A. Acquafredda [a], M.A. Panaro [a], V. Mitolo [a]

[a] *Department of Human Anatomy and Histology, University of Bari, Piazza Giulio Cesare 1, Policlinico, I-70124 Bari, Italy*
[b] *Department of Internal Medicine and Public Medicine, Section of Rheumatology, University of Bari, Piazza Giulio Cesare 1, I-70124 Bari, Italy*
[c] *Department of Internal Medicine, Immunology and Infectious Diseases, Section of Dermatology University of Bari, Piazza Giulio Cesare 1, I-70124 Bari, Italy*

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Abstract

Sjögren's syndrome (SS) is an autoimmune rheumatic disease that targets salivary and lachrymal glands, characterized by a high concentration of serum autoantibodies directed against nuclear and cytoplasmic antigens. It is known that autoantibodies can enter viable cells and this phenomenon has functional consequences including activation of apoptotic process. The objective of this work was to explore whether autoantibodies contained in IgG purified from Sjögren sera trigger apoptotic process in an experimental model represented by the human salivary gland cell line A-253. To define if the intrinsic or extrinsic pathways are activated, we examined which caspases are critical for inducing cell death. The results have demonstrated that morphological changes and DNA laddering, consistent with apoptotic cell death, occurred in A-253 cells treated with IgG from Sjögren sera. Sjögren IgG induced cleavage and activation of the effector caspase-3 and degradation of the caspase-3 substrate poly(ADP-ribose)polymerase. Both the intrinsic and extrinsic apoptotic pathways were activated, since both caspase-8 and caspase-9 cleavages occurred. In conclusion, autoantibodies contained in IgG purified from Sjögren sera mediate apoptosis of the A-253 cell line in a caspase-dependent manner.

Keywords: Apoptosis; Autoantibody; Caspase; PARP; Sjögren's syndrome

1. Introduction

The presence of serum autoantibodies, such as those directed against nuclear antigens RNP/Sm, RNP-70 Kd, dsDNA, ssDNA, poly- and mono-nucleosomes, histone-complex, and against cytoskeletal protein, such as α-fodrin, has been associated with Sjögren's syndrome (S syndrome; SS) [1–6]. S syndrome is an autoimmune rheumatic disease that targets salivary and lachrymal glands, leading to glandular atrophy characterized by keratoconjunctivitis sicca and xerostomia [7–9]. SS may occur alone, defined as primary SS, or in association with other autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus (SLE) or scleroderma, when it is defined as secondary SS [10].

Although there is a high correlation between the presence of serum autoantibodies and the development of SS, it is still unclear why cellular components, participating

* Corresponding author. Tel.: +39 080 547 8350; fax: +39 080 547 8327.
E-mail address: m.sisto@anatomia.uniba.it (M. Sisto).
[1] Both the authors contributed equally to this work.

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in important biosynthetic and structural functions, are targeted for autoimmune reactions, and the exact role of these antibodies in the pathogenesis of the disease is uncertain.

The association of apoptosis with autoantibodies production is of recent interest, in fact numerous diseases of definite or possible autoimmune aetiology have lately been shown to display abnormalities in apoptosis [11]. Apoptosis or programmed cell death (PCD) is a physiological process that assures cellular exchange; this natural machinery eliminates the apoptotic material present in blebs and apoptotic bodies [12]. Studies performed over the last years have demonstrated that the caspases, a family of cysteine-dependent, aspartate-directed proteases, play a critical role in the initiation and execution of apoptosis [13]. Caspases are synthesized as inactive zymogens that become activated by cleavage via upstream proteases and, once activated, lead to cell death via two distinct caspase-dependent pathways. The first involves the death receptor, or extrinsic pathway, which is initiated by TNF-receptor family members and involves the activation of initiator caspase-8 and/or caspase-10. The alternative intrinsic pathway, dependent on Fas and various cell stress stimuli, triggers the cell death process via mitochondrial release of Cytochrome *c* and the activation of initiator caspase-9. The active forms of caspase-8 and caspase-9 cleave and activate the downstream effectors caspase-3, caspase-6, and caspase-7 [14–16]. Once activated, caspases cleave a variety of intracellular polypeptides of cytoplasmic and nuclear localization, such as poly(ADP-ribose)polymerase (PARP) and these scissions disrupt survival pathways and disassemble architectural components of the cell, contributing to the morphological and biochemical changes that characterize apoptotic cell death. Data in literature have demonstrated that in autoimmune conditions the caspases are over activated [17] and the tolerance is broken by different factors, including an inappropriate removal of apoptotic material. This impairment promotes an antigen-driven response against intracellular antigens [18]. In the past few years, the expression of the apoptosis-regulating protein in the salivary glands of SS patients has been reported, suggesting a role for apoptotic cell death in the pathogenesis of glandular damage in this autoimmune disease [19]. Several authors have demonstrated that in SS there is an increased apoptosis of salivary gland epithelium, together with the expression of Fas and FasL, suggesting the induction of PCD through a classic Fas–FasL interaction [17]. In addition, in SS the glandular epithelium is increasingly sensitive to apoptosis, in contrast to the resistance to PCD exhibited by the infiltrating glandular lymphocytes [17,20]. At present, the mechanisms underlying the glandular tissue destruction observed in SS patients are not fully understood. Our goal in this research was to demonstrate that, in an experimental model represented by the human salivary gland cell line A-253, autoantibodies contained in IgG purified from Sjögren sera directed against cellular antigens trigger the apoptotic process. In addition, by examining which caspases are critical for inducing cell death, we show that both the intrinsic and extrinsic apoptotic pathways are activated.

2. Materials and methods

2.1. A-253 cell culture

A-253 cells from human epidermoid carcinoma of the submaxillary gland (American Type Culture Collection, number: HTB-41) were cultured in McCoy's 5a modified medium (GIBCO, CA, USA) supplemented with 10% (v/v) foetal bovine serum (FBS; SIGMA, MO, USA), 1% (v/v) antibiotic solution (100 U/ml of penicillin and 100 μg/ml of streptomycin) (SIGMA), 2 mM L-glutamine (SIGMA) and incubated in a humidified 5% CO_2–95% air incubator at 37 °C.

2.2. Patients and controls

Sera were obtained from nine healthy volunteers and nine patients with primary Sjögren's syndrome, all fulfilling the American–European Consensus Group Classification criteria for SS [1]. None of the patients examined met the clinical or serological criteria for additional connective tissue disease. None of the patients suffered from acute viral or bacterial infections at the time of the study. Informed consent for studies involving human subjects was obtained and the studies were conducted according to the tenets of the Declaration of Helsinki.

2.3. Preparation and purification of human IgG

IgG was obtained from Sjögren and healthy sera by precipitation with ammonium sulphate at 50% (w/v), followed by three washes in phosphate buffered saline (PBS) and reprecipitation with 33% (w/v) ammonium sulphate. The resulting precipitate was submitted to dialysis against phosphate buffer, pH 7.4, at 4 °C overnight. Subsequently, IgG was purified on protein G Sepharose (Amersham Pharmacia Biotech, Sweden) as recommended by the manufacturer. The IgG containing eluates, spectrophotometrically evaluated ($\lambda = 280$), were concentrated to 20 mg/ml.

2.4. ELISA autoantibodies' tests

The nine Sjögren and healthy sera, processed to obtain IgG fractions, were chosen submitting them to indirect solid phase enzyme immunoassays to detect autoantibodies against nuclear protein SS-A (Ro) and SS-B (La) (ENA combi, ELISA kit, DIAMEDIX, FL, USA), autoantibodies against nuclear antigens SSA-52 (Ro 52), SSA-60 (Ro 60), SSB (La), RNP/Sm, RNP-70 Kd, RNP-A, RNP-C, Sm-BB′, Sm-D, Sm-E, Sm-F, Sm-G, Scl-70, Jo-1, dsDNA, ssDNA, poly-nucleosome, mono-nucleosome, histone-complex, Histone H1, Histone H2A, Histone H2B, Histone H3, Histone H4, PM-Scl-100, Centromere B (ANA Detect ELISA kit, DIAMEDIX, FL, USA), and autoantibodies directed against the cytoskeletal protein α-fodrin (Anti-Alpha Fodrin detection kit, DIAMEDIX). Briefly, prediluted controls and patient samples were placed in microtiter plates precoated with the above-mentioned antigens. After 30 min incubation at room

temperature, an HRP-conjugated anti-human immunoglobulin G was added to each well to detect specific autoantibodies contained in the control and patient sera. The same bioassay was used for the detection of autoantibodies in the control and patient IgG fractions. The colorimetric reaction was performed using tetramethylbenzidine, a chromogenic substrate oxidised by HRP. After incubation for 15 min at room temperature, the optical density was measured at 450 nm with an automated microplate reader. The amount of colour was directly proportional to the concentration of autoantibodies present in samples.

2.5. Internalization of autoantibody by immunofluorescence

The internalization of SS IgG by A-253 cells was studied by fluorescence microscopy. The A-253 cells (1×10^6 cells/well) were distributed in well microculture plates (Nunc, Denmark) in which a 175-μm cellocate glass (Eppendorf, Germany) had been previously placed. The cells were incubated with SS IgG (200 μg/ml) at 37 °C for 2 h. After washing with PBS, the cells were fixed with 2% (w/v) paraformaldehyde in PBS for 10 min, and permeabilized with 0.1% (v/v) Triton X-100 in PBS for 5 min. The fixed cells were then incubated with goat anti-human IgG-FITC antibody (sc-2456; 1:200; from Santa Cruz Biotechnology, CA, USA) at room temperature for 40 min. Intracellular IgG were observed under a fluorescence microscope. To confirm the internalization of autoantibodies cells were pretreated for 2 h with cytochalasin D (15 μg/ml) and then treated with SS IgG for 2 h at 37 °C. Healthy IgG were used as control.

2.6. A-253 cell treatment

To evaluate the apoptosis, cell cultures were subjected to different treatments as follows: (1) treatment with IgG fractions (200 μg/ml) purified from healthy sera (H IgG) for 48 h; (2) treatment with IgG fractions (200 μg/ml) purified from Sjögren sera (SS IgG) for 48 h; (3) treatment with IgG (200 μg/ml) purified from healthy sera for 48 h after pre-incubation for 2 h with cytochalasin D (SIGMA) (15 μg/ml) an agent disrupting cytoskeletal filaments; and (4) treatment for 48 h with IgG purified from Sjögren sera (200 μg/ml) after pre-incubation for 2 h with cytochalasin D (15 μg/ml). Controls included both untreated cells and cells incubated for 48 h with the pro-apoptotic agent actinomycin D (SIGMA) (5 μg/ml).

2.7. Determination of apoptosis

2.7.1. Fluorescence microscopy analysis of A-253 cell apoptosis

To detect apoptotic cells, the Vibrant Apoptosis assay kit (Molecular Probes, Inc., OR, USA) was employed. This assay uses the green fluorescent non-permeant nuclear dye YO-PRO-1 that is able to enter apoptotic cells and propidium iodide (PI), that stains necrotic cells. After incubation on ice for 20 min, the cells were immediately observed under fluorescence microscopy using a green reference filter ($\lambda = 490$ nm) for YO-PRO-1 and a red reference filter ($\lambda = 530$ nm) for PI. Apoptotic cells appear green-stained, necrotic cells appear red-stained, viable cells show little or no fluorescence. To determine the optimum concentration of Sjögren IgG to employ in the experimental procedure, the same YO-PRO-1 assay was used. The A-253 cells were treated with growing concentrations of SS IgG (10, 50, 100, and 200 μg/ml) for 48 h. The A-253 cells treated with the same concentrations of healthy IgG were used as control. The percentage of apoptotic cells was determined by counting at least 100 cells.

2.7.2. DNA fragmentation of A-253 cell line

Degradation of the A-253 cell line DNA was used as an index of apoptosis. A-253 (10^7) cells from each treatment were washed in PBS and lysed in 10 mM Tris pH 7.4, 5 mM EDTA, 1% (v/v) Triton X-100 for 20 min on ice. Then 1 ml of DNAzol (Molecular Research Center Inc., OH, USA) was added to cells in the presence of proteinase K (100 μg/ml) for 30 min at 37 °C. After centrifugation at 11 000 × *g* for 20 min at 10 °C, the supernatant was collected and RNAse A (20 μg/ml) was added for 1 h at 37 °C, in order to eliminate contaminating RNA. The DNA extracted was precipitated in 100% (v/v) ethanol and centrifuged at 11 000 × *g* for 20 min. Purified DNA was dissolved in 8 mM NaOH, separated by electrophoresis in a 2% (w/v) agarose gel and visualized by ethidium bromide staining.

2.7.3. Caspase-3 activation analysis

Active caspase-3 enzyme is a heterodimer composed of two 17 kDa and two 11 kDa subunits derived from a 32-kDa proenzyme (pro-caspase-3) by cleavage at multiple aspartic acid sites. The caspase-3 precursor is firstly cleaved to produce the p11 subunit and the 20 kDa (p20) peptide. Subsequently, the p20 peptide is cleaved to generate the mature p17 subunit [21]. We adopted two different approaches to visualize the intracellular activation of caspase-3 during the apoptotic process, immunofluorescence and enzymatic assays. In the immunofluorescence assay, treated A-253 cells were fixed for 10 min in 3.7% (v/v) formaldehyde diluted in PBS plus 0.2% (v/v) Triton X-100. After treatment with 0.2% (w/v) bovine serum albumin (BSA) in PBS for 10 min to minimize non-specific absorption of antibodies, the coverslips were incubated, for 45 min at room temperature, with the rabbit anti-caspase-3 polyclonal antibody (pAb; 80806E; 1:1500; Pharmingen, Germany), specific for the 17 kDa subunit, and for 30 min with the bovine anti-rabbit IgG-FITC (sc-2365; 1:200; Santa Cruz Biotechnology). Finally, the slides were mounted and examined under a fluorescence microscope.

The enzymatic activity of caspase-3 in lysates of A-253 cells was evaluated using the CaspACE™ colorimetric assay system (Promega Corporation, WI, USA). This assay is based on the recognition of specific amino acid sequences by caspase-3 and the proteolytic cleavage of DEVD-*p*NA by caspase-3 into the chromophore *p*-nitroanline (*p*-NA). Briefly, A-253 cells, treated as described in Section 2.6, were harvested, washed twice in ice-cold PBS (pH 7.4) and then lysed with a cell lysis buffer, according to the manufacturer's

instructions. The amount of total proteins extracted was quantified by Bradford's method [22] and equal amounts (50 μg) of proteins from the total cell lysate were used in the assay. Protein lysates were transferred to a flat-bottomed microtitre plate and the reaction buffer plus the caspase-3 substrate DEVD (*N*-acetyl-Asp-Glu-Val-Asp aldehyde)-*p*NA were added. After 4 h of incubation at 37 °C, the caspase-3 activity was detected by measurement of the free *p*-nitroaniline (*p*-Na), released by cleavage of the substrate, in an absorbance spectrophotometer at 405 nm. To assess the specificity of the reaction, the competitive inhibitor of caspase-3, Z-VAD-FMK [valylalanylaspartic acid (VAD) fluoromethyl ketone (FMK), 20 mM] was added to the samples. The results are expressed as mean ± SE of nine experiments performed in duplicate.

2.7.4. Determination of caspases-8 and caspase-9 enzymatic activity

To distinguish between the two major pathways of caspase activation possibly induced by autoantibodies from Sjögren sera, we evaluated caspases-8 and caspase-9 activity in A-253 cells submitted to the treatments as indicated above. The caspase-8 and caspase-9 colorimetric protease assay kits (BioSource International Inc., CA, USA) were used.

Caspase-8, involved in the extrinsic pathway of caspase activation, exists in cells as a 55-kDa proprotein which is activated to produce p18/p17 subunits. The caspase-8 precursor is firstly cleaved to produce the p17 subunit and the p20 peptide. Subsequently, the p20 peptide is cleaved to generate the mature p18 subunit. Caspase-9, activated in the intrinsic pathway of programmed cell death, exists in cells as an inactive 46 kDa proenzyme which is cleaved to active 35 and 10 kDa subunits [21]. The kits are able to assay the amount of active caspase-8, that recognizes the IETD (Ile-Glu-Thr-Asp) amino acid sequence, and the amount of active caspase-9 that recognizes the LEHD (Leu-Glu-His-Asp) amino acid sequence. These amino acid sequences correspond to cleavage sites of the inactive 32 kDa caspase-3 precursor. The tests include a substrate for active caspase-8, IETD-*p*NA, composed of the chromophore *p*-nitroaniline (*p*-NA) and a synthetic tetrapeptide IETD, and a substrate for active caspase-9, LEHD-*p*NA, composed of the chromophore *p*-nitroaniline (*p*-NA) and a synthetic tetrapeptide LEHD. Briefly, treated cells (5×10^6) were lysed with 50 μl of the cell lysis buffer, followed by freezing and thawing cycles. For the assay, 100 μg of protein lysates was mixed with caspase assay buffers in a 96-well microtiter plate. Then, IETD-*p*NA or LEHD-*p*NA substrates were added to the wells and the microplate was incubated at 37 °C for 2 h in the dark. Upon cleavage of the substrate by active caspase-8 or caspase-9, free *p*-NA light absorbance was quantified using a microplate reader at $\lambda = 405$ nm. Comparison of the absorbance of *p*-NA from apoptotic samples or control samples allowed the determination of caspase-8 and caspase-9 activity.

2.7.5. Western blot analysis

The total cellular content of active caspase-3, caspase-8, caspase-9 and of the substrate of active caspase-3, PARP, was analysed by western blot. A-253 cells, treated as in Section 2.6, were washed twice, detached with ice-cold PBS, collected and centrifuged at $600 \times g$ for 10 min. The supernatant was removed and the pellet was incubated with lysis buffer [1% (v/v) Triton X-100, 20 mM Tris–HCl, 137 mM NaCl, 10% (v/v) glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μM leupeptin hemisulfate salt, and 0.2 U/ml aprotinin] for 30 min on ice. After incubation, the obtained lysate was vortexed and then centrifuged at $12\,800 \times g$ for 10 min; the protein concentration in the supernatant was spectrophotometrically determined by Bradford's protein assay, and the lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were diluted with sample buffer (0.5 M Tris–HCl pH 6.8, 10% (v/v) glycerol, 10% w/v SDS, 5%(v/v) β_2-mercaptoethanol, and 0.05% (w/v) bromophenol blue) and then boiled for 3 min. Proteins (25 μg/lane) and prestained standards (BioRad Laboratories, CA, USA) were loaded on 10% (PARP) or 15% (all proteins except PARP) SDS-polyacrylamide precast gels. All the experiments were carried out in duplicate. After electrophoresis, the resolved proteins were transferred from gel onto nitrocellulose membranes. A blot buffer [20 mM Tris/150 mM glycine, pH 8, 20% (v/v) methanol] was used for gel and membrane saturation and blot. The blot conditions were the following: 200 mA (constant amperage), 200 V for 110 min. After the transfer to nitrocellulose, equal loading of each lane was confirmed by Ponceau S staining. Blots were then blocked by PBS, pH 7.2, with 0.1% (v/v) Tween-20, 5% w/v non-fat dried milk for 1 h and washed three times with 0.1% (v/v) Tween-20–PBS 1× (T–PBS). Membranes were then incubated for 90 min with goat anti-caspase-3 pAb (sc-1225; 1:500), rabbit anti-PARP pAb (sc-7150; 1:500), rabbit anti-caspase-9 (sc-8355; 1:200), rabbit anti-caspase-8 (sc-7890; 1:500); all pAbs from Santa Cruz Biotechnology. Bound Ab were visualized with bovine anti-rabbit IgG-HRP (sc-2370; 1:5000; Santa Cruz Biotechnology) or rabbit anti-goat IgG-HRP (sc-2768; 1:5000; Santa Cruz Biotechnology). Bands were visualized by 3,3′-diaminobenzidine tetrahydrochloride (Fast DAB, SIGMA). The beta (β)-actin protein level was determined by western blot and used as a protein loading control.

2.8. Statistics

The data were analysed for normality using the Wilks Shapiro Test. Differences in means for paired observations were analysed by Student's *t*-test. In all instances values of $p < 0.05$ were considered as statistically significant.

3. Results

3.1. Antibodies detected in Sjögren IgG fractions

The Sjögren sera and the SS IgG fractions resulted positive for the presence of anti-SS-A, anti-SS-B, anti-nuclear antigens. Seven of the SS sera and SS IgG fractions were positive for anti-α-fodrin autoantibodies. Healthy sera and healthy IgG

Table 1
ELISA detection of autoantibodies in IgG fractions of nine patients with primary Sjögren syndrome

SS IgG sample, *N*	SS IgG anti-SS-A (Ro) (U/ml)	Anti-SS-A results positive > 15 U/ml	SS IgG anti-SS-B (La) (U/ml)	Anti-SS-B results positive > 15 U/ml	SS IgG O.D. ANA[a]	O.D. cut-off	ANA[a] index	ANA[a] results positive > 1.2	SS IgG anti-α-fodrin (U/ml)	Anti-α-fodrin results positive > 10 U/ml
1	18	Positive	31	Positive	1.294	0.435	2.97	Positive	11.7	Positive
2	25	Positive	28	Positive	1.394	0.435	3.2	Positive	13.4	Positive
3	28	Positive	25	Positive	2.5	0.435	5.74	Positive	16.8	Positive
4	22	Positive	24	Positive	2.3	0.435	5.28	Positive	17.9	Positive
5	18	Positive	22	Positive	1.15	0.435	2.64	Positive	14.2	Positive
6	23	Positive	22	Positive	0.99	0.435	2.27	Positive	6.4	Negative
7	25	Positive	27	Positive	1.2	0.435	2.75	Positive	12.8	Positive
8	28	Positive	35	Positive	0.88	0.435	2.02	Positive	11.9	Positive
9	22	Positive	30	Positive	0.75	0.435	1.72	Positive	5.9	Negative

[a] ANA corresponds to autoantibodies against Ro 52, Ro 60, La, RNP/Sm, RNP-A, RNP-C, Sm-BB′, Sm-D, Sm-E, Sm-F, Sm-G, Scl-70, jo-1, dsDNA, ssDNA, poly-nucleosome, histone-complex, Histone H1, Histone H2A, Histone H2B, Histone H3, Histone H4, PM-Scl-100, centromere beta.

fractions were negative for all autoantibodies tested (autoantibodies detected are listed in Table 1).

3.2. Antibodies internalization by the A-253 cells

To examine the uptake of Sjögren IgG by A-253 cells after 2 h incubation, an immunofluorescence assay was performed using a goat anti-human IgG-FITC antibody. The cells treated with SS IgG (Fig. 1A) showed an higher fluorescence, located in the cytoplasm or inside the nucleus, than control cells, treated with healthy IgG (Fig. 1B), that showed a low fluorescence signal distributed in a homogeneous manner inside the cells. Pretreatment with cytochalasin D blocked the SS IgG (Fig. 1C) and the healthy IgG internalization (data not shown). Cells treated with the goat anti-human IgG-FITC antibody alone did not show any fluorescence and, at the same time,

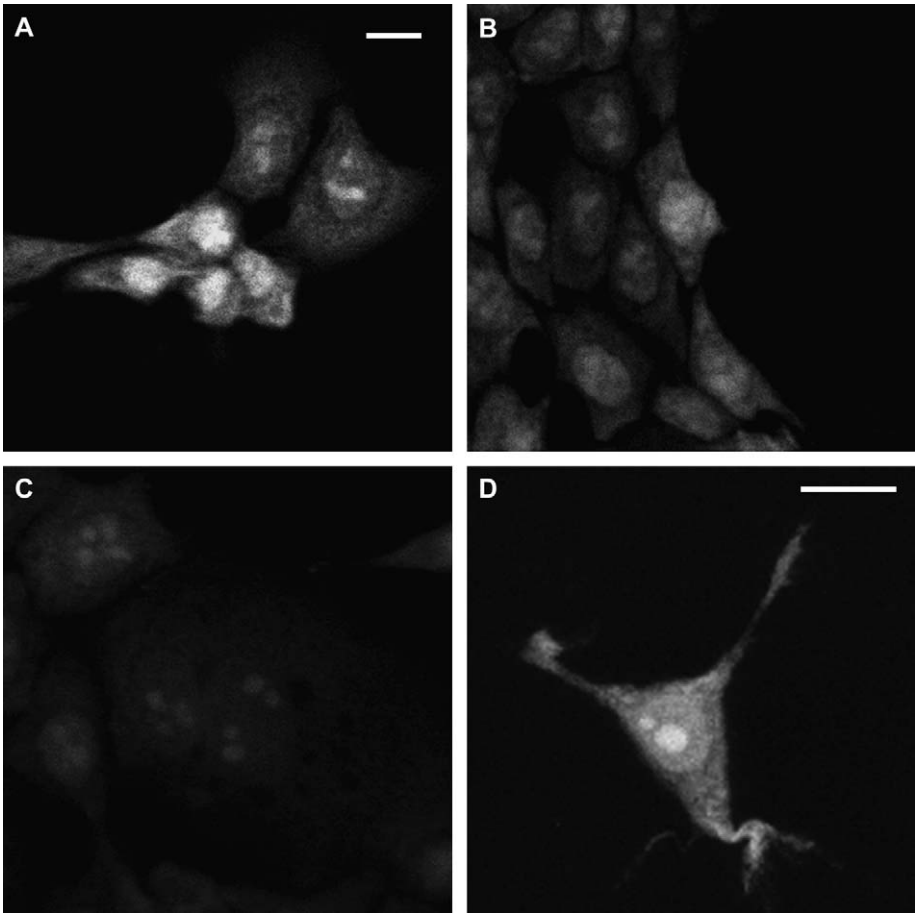

Fig. 1. Internalization of Sjögren IgG by A-253 cells detected by fluorescence microscopy. The SS IgG internalization was revealed using a goat anti-human IgG-FITC antibody. Cells treated with SS IgG (A); cells treated with healthy IgG (B); cells pretreated with cytochalasin D and SS IgG (C); higher magnification of SS IgG-treated cell (D). Scale bar = 10 μm. The results are representative of nine experiments.

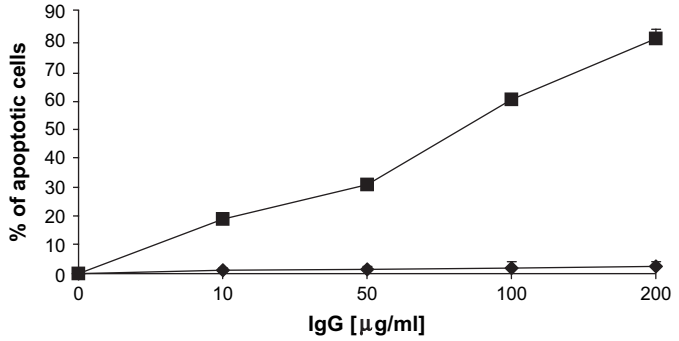

Fig. 2. Percentage of apoptotic cells at growing concentrations of Sjögren IgG. The percentage of apoptotic cells was assessed using the YO-PRO-1 assay kit. The A-253 cells treated with growing concentrations of Sjögren IgG (■) and healthy IgG (◆) were observed under fluorescence microscopy (mean ± SE of nine experiments).

cells treated without permeabilization buffer prior to the incubation of the secondary antibody showed positive membrane labelling (data not shown).

3.3. Effect of Sjögren IgG concentration on A-253 cells apoptosis

The nuclear morphology of A-253 cells, treated with growing concentrations of Sjögren IgG, was analysed by the Vybrant Apoptosis assay kit. Fig. 2 shows the percentage of apoptotic A-253 cells at different concentrations of Sjögren IgG. When the concentration of 200 μg/ml was used the percentage of apoptotic cells was 82 ± 3.2. The concentration of 200 μg/ml resulted optimum and was employed in all the experimental procedures. Healthy IgG had no effect on apoptotic morphology at the same concentration.

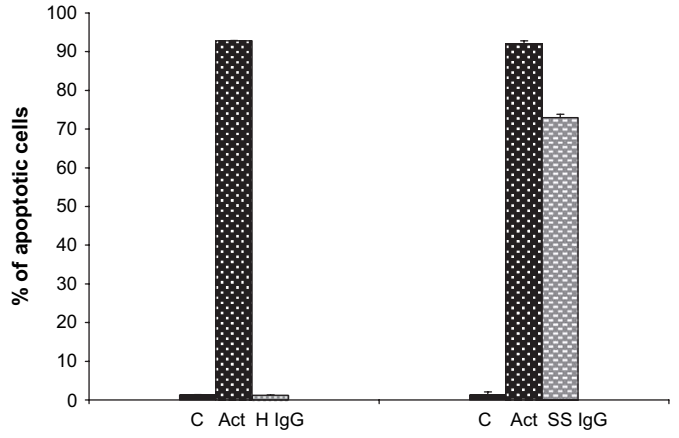

Fig. 3. Apoptotic staining in A-253 cells treated with Sjögren IgG. The percentage of apoptotic cells was assessed using the YO-PRO-1 assay kit. The A-253 cells treated with IgG purified from Sjögren and healthy sera were observed under fluorescence microscopy. Controls included untreated cells and cells treated with the pro-apoptotic agent actinomycin D. The *y*-axis shows the percentage of apoptotic cells. C, untreated control cells; Act, control cells treated with actinomycin D; H IgG, cells treated with IgG purified from healthy sera; SS IgG, cells treated with IgG purified from Sjögren sera (means ± SE of nine experiments).

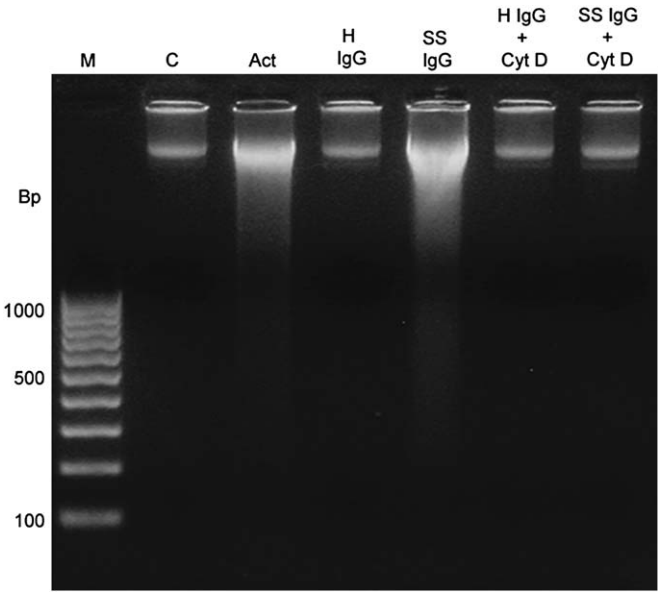

Fig. 4. SS IgG induce DNA laddering in A-253 cells. The cells were treated with IgG purified from healthy sera (lane H IgG), IgG purified from Sjögren sera (lane SS IgG), cytochalasin D plus healthy IgG (lane H IgG + Cyt D), cytochalasin D plus Sjögren IgG (lane SS IgG + Cyt D). Untreated cells (lane C) and cells treated with actinomycin D (lane Act) were used as control. The molecular weight standard was run in lane M. DNA from these cells was extracted and analysed by 2.0% agarose gel electrophoresis.

3.4. Morphological analysis of apoptotic A-253 cells

For analysis of the nuclear morphology, A-253 cells, stained with the Vybrant Apoptosis assay kit, were observed under fluorescence microscopy. As shown in Fig. 3, the percentage of apoptotic A-253 cells treated with IgG purified from Sjögren sera (78.9 ± 0.7) was significantly higher ($p < 0.01$) than the percentage of apoptotic cells observed in untreated control cells (1.2 ± 0.1). No significant difference ($p > 0.05$) was revealed between the percentage of apoptotic A-253 cells treated with IgG purified from healthy sera (1.3 ± 0.1) and control cells (1.2 ± 0.1).

3.5. IgG purified from Sjögren sera induce DNA fragmentation

DNA fragmentation analysis in A-253 cells treated as described in Section 2 yielded these results: DNA fragmentation was observed in cells treated with the pro-apoptotic agent actinomycin D, as in cells treated with IgG purified from Sjögren sera. Moreover, SS IgG-induced DNA fragmentation was prevented by preliminary treatment with cytochalasin D. No DNA fragmentation was observed in untreated control cells and in cells treated with IgG purified from healthy donors (Fig. 4).

3.6. IgG purified from Sjögren sera induce caspase-3 activation

In the immunofluorescence assay, the cells in which caspase-3 was activated appeared green-stained. Active caspase-3 was observed in actinomycin D-treated A-253 cells

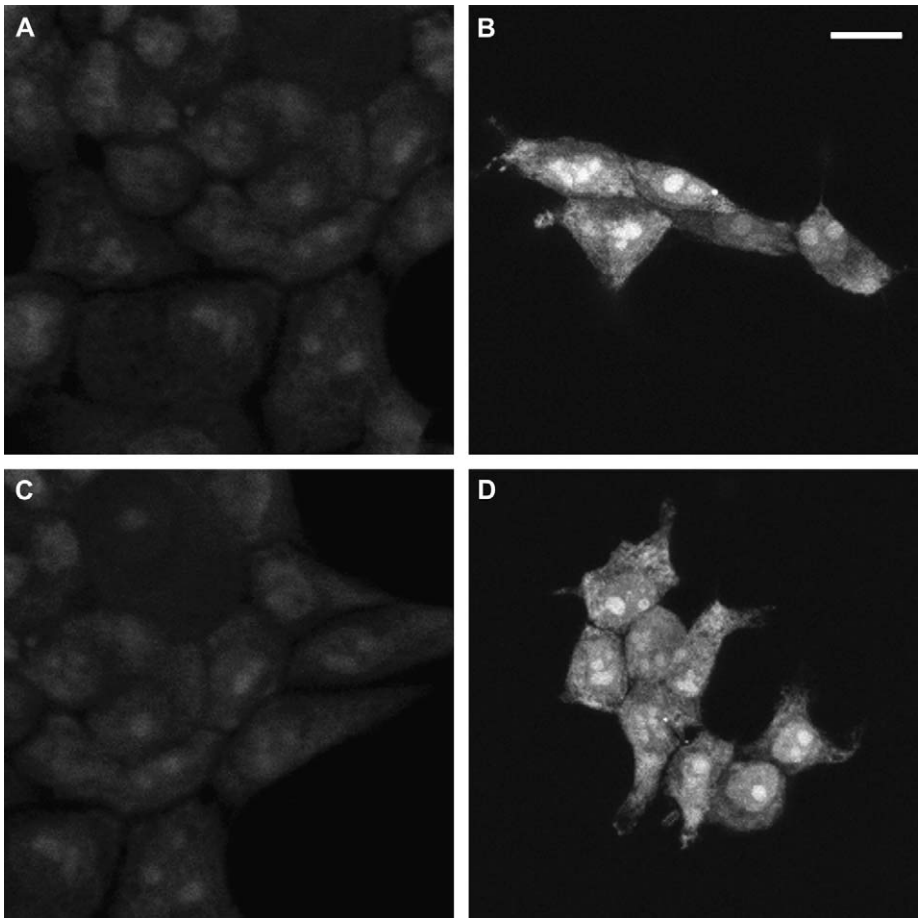

Fig. 5. Fluorescence microscopy of A-253 cells stained with rabbit anti-caspase-3 pAb using fluorescein isothiocyanate (FITC)-conjugated bovine anti-rabbit IgG as detection antibody. A-253 untreated control cells (A) and cells treated with IgG purified from healthy donors sera (C) did not show intracellular caspase-3 activation. Actinomycin D-treated A-253 cells (B) and cells treated with IgG purified from Sjögren sera (D) showed intracellular activation of caspase-3 (in the figure, green-stained cells appear white). Scale bar = 10 μm. The results are representative of nine experiments.

(Fig. 5B) and in cells treated with IgG purified from Sjögren sera (Fig. 5D). By contrast, A-253 untreated control cells (Fig. 5A) and cells treated with IgG purified from healthy sera (Fig. 5C) did not show intracellular caspase-3 activation. Moreover, pretreatment with cytochalasin D prevented caspase-3 activation induced by SS IgG cell treatment (data not shown).

Fig. 6 shows the active caspase-3 proteolytic activity measured by the CaspACE™ colorimetric assay system. A significantly higher ($p < 0.01$) caspase-3 enzymatic activity was observed in A-253 cells treated with IgG from Sjögren sera in comparison with untreated control cells. No significant differences in caspase-3 activity were observed between A-253 cells treated with IgG from healthy sera and untreated control sera ($p > 0.05$). A significant ($p < 0.01$) reduction in caspase-3 enzymatic activity was observed when A-253 cells were pretreated with cytochalasin D and then with SS IgG in comparison with cells treated with SS IgG alone. The specificity of the reaction, assessed using the competitive inhibitor of caspase-3, Z-VAD-FMK was reliable because treatment with this substance blocked DEVD-*p*NA cleavage (data not shown).

3.7. IgG purified from Sjögren sera induce activation of both caspase-8 and caspase-9

Fig. 7 shows the active caspase-8 (Fig. 7A) and the active caspase-9 (Fig. 7B) proteolytic activity measured by the caspase-8 and caspase-9 colorimetric protease assay kits. A-253 cells treatment with IgG purified from Sjögren sera led to the activation of both of these caspases, involved in different apoptotic pathways. In fact, a significantly higher ($p < 0.01$) caspase-8 enzymatic activity was observed in IgG from Sjögren sera-treated A-253 cells in comparison with untreated control cells. No significant differences in caspase-8 activity were observed between A-253 cells treated with IgG from healthy sera and untreated control sera ($p > 0.05$). A significant ($p < 0.01$) reduction in caspase-8 enzymatic activity was observed when A-253 cells were treated with cytochalasin D plus SS IgG, in comparison with cells treated with SS IgG alone. The same results were observed for caspase-9 activation, in fact, SS IgG treatment of A-253 cell line led to the activation of caspase-9, and the enzymatic activity of caspase-9 was significantly higher ($p < 0.01$) than in untreated control cells and cells treated with H IgG. Moreover, caspase-9

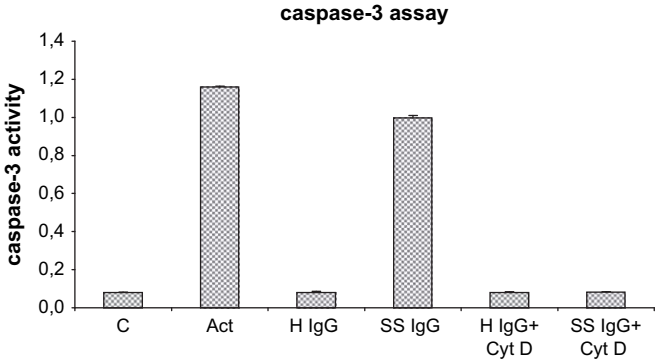

Fig. 6. Caspase-3 activity in A-253 cells. The enzymatic activity of caspase-3 was measured in lysates of A-253 treated with IgG purified from healthy sera (H IgG), IgG purified from Sjögren sera (SS IgG), cytochalasin D plus healthy IgG (H IgG + Cyt D) and cytochalasin D plus Sjögren IgG (SS IgG + Cyt D). Untreated cells (C) and cells treated with actinomycin D (Act) were used as controls. The *y*-axis shows the amount of free *p*-nitroaniline (*p*-NA) released from the caspase-3 specific substrate DEVD-*p*NA. The data are representative of nine experiments and the results are expressed in arbitrary units.

activation following SS IgG treatment was significantly ($p < 0.01$) reduced when cytochalasin D was used.

3.8. IgG purified from Sjögren sera induce proteolytic cleavage of initiator and executor caspases

Cleavage of the initiator caspases-8 and caspase-9 and of the effector caspase-3 and active caspase-3 substrate, PARP, was analysed by western blot. Western blot analysis of caspase-3 protein activation was performed using the anti-caspase-3 antibody, specific for peptide mapping at the C-terminus of the caspase-3 subunit p20, as a probe. The antibody recognizes both the caspase-3 precursor and the active caspase-3 subunit p20. The presence of the 20 kDa band was evaluated in A-253 untreated control cells and in cells treated with actinomycin D, IgG purified from Sjögren sera, and IgG purified from healthy sera. The band corresponding to the p20 subunit detected in actinomycin D-treated cells was evident in SS IgG-treated cells as well. Only the caspase-3 precursor was detectable in untreated control cells and in cells treated with H IgG (Fig. 8 panel A). To confirm caspase-3 activation, the expression of the 89 kDa fragment of PARP, resulting from cleavage of endogenous 116 kDa PARP by active caspase-3, was analysed. In A-253 control cells and in cells treated with IgG from healthy donors, the full length 116 kDa PARP was expressed. Treatment of A-253 with actinomycin D and with IgG from Sjögren patients induced the cleavage of the full length PARP in the 89 kDa subunit (Fig. 8 panel B). Western blot analysis of the caspase-8 protein activation was performed using the anti-caspase-8 antibody, specific for the p20 subunit, which is able to recognize both the inactive pro-caspase-8 and the active p20 fragment. Pro-caspase-9 cleavage was revealed using the anti-caspase-9 antibody, specific for the p35 active subunit. As shown in Fig. 9, SS IgG treatment induced pro-caspase-8 (Fig. 9 panel A) and pro-caspase-9 (Fig. 9 panel B) activation. No proteolytic cleavage of the initiator caspases was observed following A-253 cells treatment with IgG purified from healthy sera.

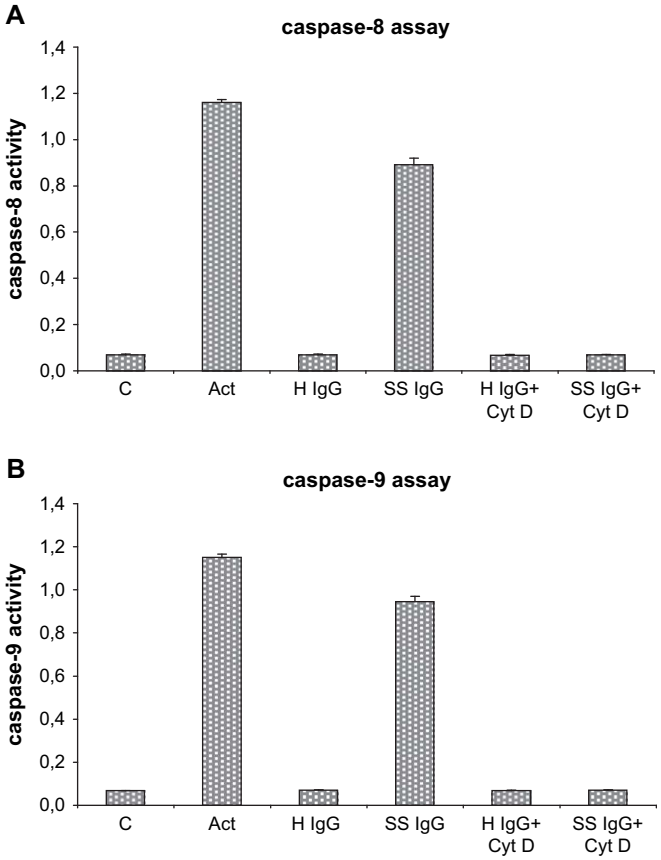

Fig. 7. Caspase-8 and caspase-9 activity in A-253 cells. The enzymatic activity of caspase-8 (A) and caspase-9 (B) was measured in lysates of A-253 cells treated with IgG purified from healthy sera (H IgG), IgG purified from Sjögren sera (SS IgG), cytochalasin D plus healthy IgG (H IgG + Cyt D) and cytochalasin D plus Sjögren IgG (SS IgG + Cyt D). Untreated cells (C) and cells treated with actinomycin D (Act) were used as controls. In (A), the *y*-axis shows the amount of free *p*-nitroaniline (*p*-NA) released from the caspase-8 specific substrate IETD-*p*NA. In (B), the *y*-axis shows the amount of free *p*-nitroaniline (*p*-NA) released from the caspase-9 specific substrate LEHD-*p*NA. The data are representative of nine experiments and the results are expressed in arbitrary units.

4. Discussion

This study was undertaken to determine whether autoantibodies against several antigens contained in IgG purified from Sjögren sera trigger apoptosis in human salivary gland cell line A-253. Our findings demonstrate that in A-253 cells autoantibodies induce (1) the activation of both the intrinsic and the extrinsic apoptotic pathways; (2) then the cleavage and activation of effector caspase-3; and, finally, (3) morphological changes typical of apoptosis. On the contrary, IgG purified from healthy sera have no effects on A-253 cells.

The ability of some autoantibodies to enter viable cells and react with cellular antigens is accepted from several authors. In varying experimental models, both in vitro and in vivo, it

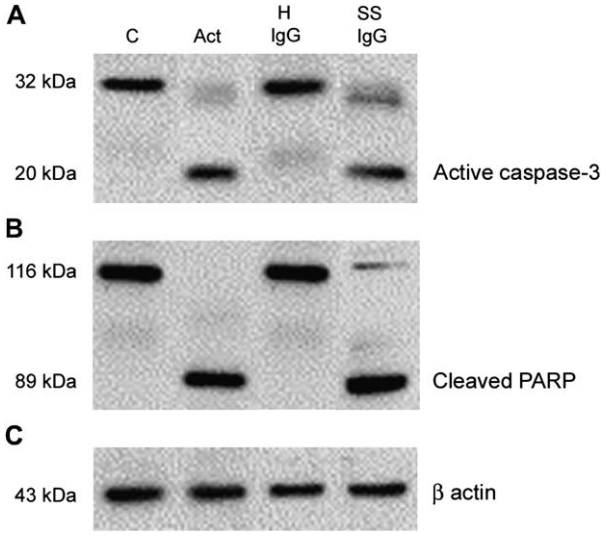

Fig. 8. Proteolytic cleavage of caspase-3 and PARP in A-253 cells. Cell lysates of A-253 cells were collected, separated by SDS-PAGE (15% for caspase-3 and 10% for PARP) and transferred onto a nitrocellulose membrane for western blot analysis. Blots were probed with polyclonal antibodies for caspase-3 (panel A), PARP (panel B) and β-actin (panel C). Act, actinomycin D-treated cells; C, untreated control cells; H IgG, A-253 treated with IgG purified from healthy sera; SS IgG, A-253 treated with IgG purified from Sjögren sera.

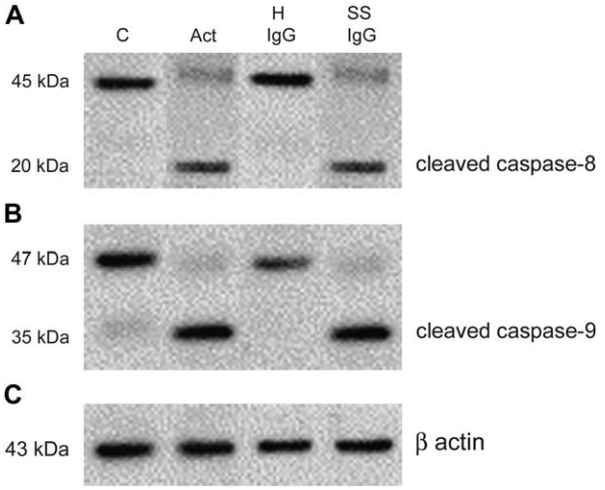

Fig. 9. Proteolytic cleavage of caspase-8 and caspase-9 in A-253 cells. Cell lysates of A-253 cells were collected, separated by SDS-PAGE (15%) and transferred onto a nitrocellulose membrane for western blot analysis. Blots were probed with polyclonal antibodies for caspase-8 (panel A), caspase-9 (panel B) and β-actin (panel C). Act, actinomycin D-treated cells; C, untreated control cells; H IgG, A-253 treated with IgG purified from healthy sera; SS IgG, A-253 treated with IgG purified from Sjögren sera.

has been proven that antibodies to intracellular constituents can enter living cell types from many tissues, causing various functional alterations [23–36]. Autoantibodies that penetrate live cells include polyclonal and monoclonal anti-DNA [24,37–39], anti-ribonucleoprotein (RNP) [23,40], anti-Ro [41,42], anti-La [43], anti-proteinase 3 [44], anti-synaptosomal [45], anti-neuronal [46,47], anti-ribosomal protein P [48], and anti-α-fodrin [49], a gene protein product of recent identification. In particular, it has been reported that polyclonal IgG anti-RNP isolated from autoimmune patients penetrate live human peripheral blood mononuclear cells [40,23], keratinocytes [50] and epithelial cells [38]. Furthermore, human polyclonal anti-DNA IgG isolated from systemic lupus erythematosus serum penetrate and localize in the cell nucleus of peripheral blood T lymphocytes [28,51,52], human and murine renal tubular cells [53].

Several mechanisms have been investigated for the penetration of autoantibodies [23,53–55]; it has been proposed that different antibodies may preferentially use distinct penetration pathways [27,53], including the engagement of Fc receptors on cells, originally described by Alarcón-Segovia et al. [23], or the cross-reaction of antibodies with proteins located on or near the cell surface such as myosin 1, a 110-kDa cell surface receptor, in the case of monoclonal anti-DNA antibodies [56–58].

Several findings suggest that there is a relationship between the binding and penetration of autoantibodies into different cell types and the initiation of events leading to various functional cellular alterations. Autoantibodies can thereby modify cell functions, arrest the progression of the cell cycle and abrogate the expression of some genes [33]. One of the most interesting effects of autoantibody penetration is the induction of programmed cell death [24,28,35,46,47].

Growing evidence suggests that the deregulation of apoptosis is involved in autoimmunity. Autoimmune diseases are characterized by aberrations in the repertoire of lymphocytes that infiltrate target organs in response to autoantigens arising from inappropriate apoptosis of target cells [59]. Often, the presence of autoreactive lymphocytes is involved in the progression of the autoimmune diseases triggered by autoantibodies' penetration [60–62]. Numerous investigations have focused on the role of CD95/Fas in organ-specific autoimmunity as shown in Hashimoto's thyroiditis [63], Grave's disease [64], insulin-dependent diabetes mellitus [65] and systemic lupus erythematosus [66]. In SLE there is evidence of an increase of the soluble Fas mediator, that would inhibit the apoptotic process, but this feedback is not absolutely decisive for autoimmune cell survival, because a high level of lymphocyte apoptosis was concurrently found [67].

Recently, research advances have supported the idea that, once they have penetrated cells, autoantibodies can be directly pathogenic and directly trigger the apoptotic pathway, depending on their antigen specificity [68]. Shiraga and Adamus showed that anti-retinal autoantibodies of the paraneoplastic retinopathy syndrome, specific to recoverin and to enolase, but not normal antibodies, can induce activation of the apoptotic process via the intrinsic pathway in living retinal cells [69]. Furthermore, DNA-specific autoantibodies can enter cultured mesangial cells [70], endothelial cells [71], and lymphoid cells [33], causing apoptosis.

Apoptosis also seems to play a central role in the pathogenesis of Sjögren's syndrome. S syndrome has been demonstrated to proceed along two pathological phases: an early phase in which the salivary and lachrymal tissues undergo inappropriate PCD and a later phase of lymphocyte infiltration and autoimmune aggression, in which further progressive glandular destruction and functional disability occur [72].

Apoptotic death in SS may be mediated through the tumor necrosis factor-alpha receptor [73], CD95 [74,75] or the perforin pathway [3]. Recent observations have detected increased proteolytic activity in salivary epithelial cells, including that of the caspases, enzymes involved in the intracellular PCD, supporting the concept that excessive cell death contributes to the pathogenesis of this disease [76].

At present, in Sjögren's syndrome, there are no evidences of any correlation between autoantibody penetration of salivary epithelial cells and a direct role in causing apoptosis. In this report, we demonstrate that, in our experimental model represented by a human salivary gland cell line A-253, autoantibodies contained in IgG purified from Sjögren sera, once penetrated into cells, are able to trigger cell death through apoptotic mechanisms.

Using YO-PRO-1 staining, we observed morphological changes consistent with apoptotic cell death in A-253 cells treated with SS IgG. DNA fragmentation into variable length oligonucleosomal fragments is one of the typical features of apoptosis. Characteristic DNA laddering, observed in A-253 cells treated with SS IgG, confirmed cellular apoptosis. Inhibition of autoantibody internalization, by blocking endocytosis using cytochalasin D, an agent that disrupts cytoskeletal filaments, conserved the DNA integrity. In addition, to distinguish between the two major pathways of caspase activation, we evaluated caspase-3, caspase-8 and caspase-9 proteolytic cleavage and activation in A-253 cells treated with SS IgG. In A-253 cells, the treatment with SS IgG-induced cleavage and activation of effector caspase-3, a key and pivotal executioner protease, confirmed by cleavage of caspase-3 substrate poly(ADP-ribose)polymerase (PARP) that is cleaved and inactivated when caspase-3 is activated. Colorimetric and western blot analyses demonstrated that both the intrinsic and extrinsic pathways were activated, since both caspase-8 and caspase-9 cleavage occurred. Pretreatment of A-253 cells with cytochalasin D inhibited caspase-3, caspase-8, and caspase-9 cleavage and activation, confirming that this effect was caused by autoantibody penetration. In the same experimental condition, IgG purified from sera of healthy donors did not have the apoptotic effect that SS IgG had.

In conclusion, we have shown that autoantibodies contained in IgG purified from Sjögren sera mediate apoptosis of a salivary gland cell line in a caspase-dependent manner, in which both the extrinsic and intrinsic pathways play a role. This may be an important step towards finding more appropriate measures for the treatment of Sjögren's syndrome. A better understanding of the events responsible for the glandular damage occurring in this autoimmune disease may allow new therapeutic strategies to be instituted to prevent epithelial cell death and the consequent impairment of the secretory function.

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