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Fcγ receptors mediate internalization of anti-Ro and anti-La autoantibodies from Sjögren's syndrome and apoptosis in human salivary gland cell line A-253

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BACKGROUND: The presence of serum anti-Ro and anti-La autoantibodies directed against the ribonucleoproteins Ro and La has been associated with Sjögren's syndrome (SS), an autoimmune rheumatic disease that targets salivary and lachrymal glands. There is increasing evidence of the direct involvement of autoantibodies in the pathogenesis of tissue injury and correlation of their presence with clinical manifestations in SS. The focus of this work was to explore the cellular apoptotic pathway triggered by binding and penetration of anti-Ro and anti-La autoantibodies in human salivary gland cell line A-253 and to identify the membrane receptors through which anti-Ro and anti-La could exert their effect.

METHODS: Anti-Ro and anti-La autoantibodies were purified from IgG fractions, obtained from eleven healthy volunteers and patients with primary Sjögren's syndrome, using Sepharose 4B-Ro and Sepharose 4B-La affinity columns. Flow cytometry, RT-PCR, western blot and confocal microscopy analysis were used to visualize the FCγRI, FCγRII and FCγRIII receptors on the A-253 cell membrane. DNA laddering and western blot analysis of caspases activation were studied to evaluate in A-253 cells treated with anti-Ro and anti-La autoantibodies.

RESULTS: The results yeilded the evidence of the presence of members of the Fcγ receptors (FcγRs) family on the cell membrane of the human salivary gland cell line A-253. Furthermore, we demonstrated that, in the A-253 cell line, anti-Ro and anti-La autoantibodies can access the cells probably through Fcγ receptors, and trigger apoptotis.

CONCLUSIONS: We conclude that anti-Ro and anti-La autoantibodies have pathogenic effects that could depend on binding to Fcγ receptors.

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Introduction

Sjögren's syndrome is an autoimmune disease that manifests primarily in the salivary and lachrymal glands, characterized by oral and ocular dryness, which is why it is known as the sicca syndrome. It occurs alone (primary SS) or in association with other rheumatic disease (secondary SS), most commonly with systemic lupus erythematosus or rheumatoid arthritis. Currently used criteria to classify patients affected by Sjögren's syndrome depend on the demonstration of dry eyes and mouth, on focal inflammation findings at salivary gland biopsy and on the presence of autoantibodies in the serum (1, 2). The presence of serum autoantibodies directed against a range of cellular components including DNA, nucleosomes and the protein constituents of the ribonucleoproteic complex has been associated with Sjögren's syndrome (1–6). Nevertheless, the best-defined antibodies in SS are those reacting with the ribonucleoprotein antigens SS-A (Ro) and SS-B (La). Anti-Ro antibodies are found in about 70–90%, and anti-La in approximately the same frequency, of patients with primary SS (7), and the presence of these autoantibodies coincides with a high frequency of extraglandular manifestations (8). The Ro (9, 10) and La (11) proteins are antigens that have been well characterized, cloned and sequenced. Ro and La have been shown to be ribonucleoproteins (12) and the data suggest that Ro is involved in the regulation of the translational fate of ribosomal protein mRNAs, and in quality control or discard pathways for 5S rRNA production (13, 14), acting as a transcription factor regulating gene expression (15). The La protein is involved in the initiation and termination of RNA polymerase III transcription, in translational control, and in regulating viral replication (16, 17).

At present, the mechanisms underlying the glandular tissue destruction observed in SS patients are still a matter of speculation. In recent years several studies have focused on autoantibodies and autoimmune disease. There is increasing evidence of the direct involvement of autoantibodies in the pathogenesis of tissue injury and of the correlation of their presence with clinical manifestations in SS (18–20). However, the exact role of autoantibodies in the pathogenesis of SS remains controversial. Several findings suggest that there is a relationship between binding and penetration of autoantibodies in the different cell types (21, 22) and the initiation of events leading to various functional cellular alterations (23–25). Autoantibodies can thereby modify cell functions, arrest progression of the cell cycle (26) and abrogate the expression of some genes (27, 28).

Programmed cell death (apoptosis) has been clearly implicated in salivary gland destruction in Sjögren's syndrome (29–31). Activation of a cascade of proteolytic enzymes called caspases (a family of cysteine-dependent, aspartate-directed proteases) is an important step in the apoptotic process (32). The two best known pathways of caspases activation are the extrinsic pathway that involves the activation of initiator caspase-8 and⁄or -10, and the intrinsic pathway, characterized by the activation of initiator caspase-9. The active forms of caspase-8 and caspase-9 activate the effectors caspases-3, -6 and -7 (33). In our recent *in vitro* study, we succeeded in showing in an experimental model, namely the human salivary gland cell line A-253, that autoantibodies contained in IgG purified from Sjögren sera penetrate cells and are able to trigger cell death through apoptotic mechanisms (18). There has been no previous investigation of a direct correlation between the serum presence of anti-Ro and anti-La autoantibodies in Sjögren's syndrome patients and the triggering of the apoptotic process in salivary glands. Furthermore, although our previous work (18) demonstrated that autoantibodies are able to penetrate the salivary gland cell line, the mechanism through which the autoantibodies gain access to cells are still unclear. The focus of this paper was to gain a greater insight into the cellular dysfunction mechanisms induced by binding and penetration of anti-Ro and anti-La autoantibodies and to identify the membrane receptors through which anti-Ro and anti-La could exert their effect. Herein, we have demonstrated that in the salivary gland cell line A-253, autoantibodies anti-Ro and anti-La, isolated from Sjögren IgG, can access cells, probably via Fcγ receptors, and once penetrated, cause cell death through apoptotic mechanisms.

Materials and methods

Cell cultures

A-253 cells from human epidermoid carcinoma of the submaxillary gland (American Type Culture Collection, USA, number: HTB-41) were cultured in McCoy's 5a-modified medium (Gibco, NY, USA) supplemented with 10% (v⁄v) foetal bovine serum (FBS; Sigma, St Louis, MO, USA), 1% (v⁄v) antibiotic solution (100 U⁄ml penicillin, 100 μg⁄ml streptomycin; Sigma), 2 mM L-glutamine (Sigma) and incubated in a humidified 5% CO_2–95% air incubator at 37°C. Human monocyte⁄macrophage U-937 cells from histiocytoma (American Type Culture Collection, number: CRL-1593.2) were cultured as described by Lisi et al. (34).

Patients and controls

Sera were obtained from 11 healthy volunteers and 11 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.

Preparation and purification of human IgG

Immunoglobulin G was obtained from 20 ml of each Sjögren and healthy sera by precipitation with ammonium sulphate at 50% (w⁄v), followed by three washes in phosphate-buffered saline (PBS; Gibco) and reprecipitation with 33% (w⁄v) ammonium sulphate (Sigma). 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, Buckinghamshire, UK) as recommended by the manufacturer. The IgG containing eluates, spectrophotometrically evaluated (λ = 280), were concentrated to 20 mg⁄ml. The purity of the IgG fractions collected was determined by mini sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel.

ELISA anti-Ro and anti-La autoantibodies assay

An indirect solid phase enzyme immunoassays to detect autoantibodies against nuclear protein Ro and La (Is® anti-SSA and Is® anti-SSB, ELISA kit, Diamedix, Miami, FL, USA) was performed to select the 11 Sjögren IgG-positive for the presence of both anti-Ro and anti-La autoantibodies. Healthy IgG fractions, negative for the autoantibodies tested, were used as control. Data are listed in Table 1.

Anti-Ro and anti-La purification

Anti-Ro and anti-La autoantibodies were purified from Sjögren IgG fractions (20 mg⁄ml) using Sepharose 4B-Ro and Sepharose 4B-La affinity columns as recommended by the manufacturer. The affinity columns were prepared by coupling 5 mg of Ro and La antigens (ImmunoVision, Springdale, AR, USA) per 1 ml of CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech). Sjögren IgG was loaded and washed with 10 volumes of starting buffer. The anti-Ro and the anti-La antibodies were eluted with 0.1 M glycine (Sigma), pH 2.5, and neutralized immediately. The purity of the anti-Ro and anti-La autoantibodies was determined by SDS-PAGE. The autoantibodies obtained were concentrated to 200 μg⁄ml by ultrafiltration methods; the

Table 1 ELISA detection of anti-Ro and anti-La autoantibodies in IgG fractions of 11 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*
1	18	Positive	31	Positive
2	25	Positive	28	Positive
3	28	Positive	25	Positive
4	22	Positive	24	Positive
5	18	Positive	22	Positive
6	23	Positive	22	Positive
7	25	Positive	27	Positive
8	28	Positive	35	Positive
9	22	Positive	30	Positive
10	27	Positive	28	Positive
11	24	positive	26	positive

concentration in the final product was estimated using Bradford's methods (35). Anti-Ro and anti-La autoantibodies were used at concentration of 20 μg/ml in all experimental procedures. To confirm the results obtained using the anti-Ro and anti-La antibodies separated by affinity chromatography, the human antibody against Ro/SS-A and the human antibody against La/SS-B (ImmunoVision) at concentrations of 20 μg/ml were used as control.

$F(ab')_2$ from anti-Ro and anti-La autoantibodies preparation

For $F(ab')_2$ fragments preparation, the anti-Ro and anti-La autoantibodies were digested with pepsin (Sigma) in 0.2 M acetate buffer, pH 4.5, at 37°C overnight. The digestion was terminated by dialyzing against PBS, pH 7.4, overnight and the digest was passed through protein A columns to remove undigested auto-Abs or Fc fragments. The quality of the digestion and the concentration of $F(ab')_2$ was determined by SDS-PAGE.

Flow cytometry and confocal microscopy analysis of membrane receptors FCγ RI, FCγ RII and FCγ RIII in A-253 cells

Two different approaches were adopted to visualize the receptors FCγRI, FCγRII and FCγRIII on the A-253 cell membrane. For the FACS analysis cells (1×10^5) were incubated with the mouse antihuman FCγRI biotin (MCA756B), mouse antihuman FCγRII biotin (MCA1075B) and mouse antihuman FCγRIII biotin (MCA1193B; all Abs were from Serotec, Ltd, Oxford, UK) and thereafter with streptavidin-RPE (STAR4A, Serotec). Cell surface expression of FCγRI, FCγRII and FCγRIII was analysed by Becton Dickinson (BD, Becton Dickinson, Frankline Lakes, NJ, USA) FACSCanto™ II flow cytometer and BD FACS DIVA software. For the immunofluorescence assay, the A-253 cells (1×10^6 cells/well) were distributed in well microculture plates (Nunc, Roskilde, Denmark) after placement of a 175 μm-cellocate glass (Eppendorf, Hamburg, Germany). The cells were fixed with 2% (w/v) paraformaldehyde (Sigma) in PBS for 10 min, and then treated with biotinylated antihuman FCγRI, biotinylated antihuman FCγRII and biotinylated antihuman FCγRIII (all antibodies were purchased from Serotec) for 90 min at room temperature. Streptavidin (FITC; Chemicon, Temecula, CA, USA) was used for FITC secondary detection. The cells were observed using a confocal laser scanning microscopy system (Leica, TCS-SP2, Wetzlar, Germany) using a $\lambda = 488$ nm Argon-Crypton laser for FITC. The monocytic cell line U-937, that expresses all members of FcγRs on its membrane (36, 37), was used as control for the entire experimental procedure.

Western blot and RT-PCR analysis of membrane receptors FCγRI, FCγRII and FCγRIII in A-253 cells

For the immunoblot analysis, the membrane protein extract of A-253 cells was obtained using the Proteo-Extract® Subcellular Proteome Extraction Kit (Merck KGaA, Whitehouse Station, NJ, USA), as described by the manufacturer, that enables the differential extraction of proteins according to their subcellular localization. The protein concentration in the membrane fraction was spectrophotometrically determined by Bradford's protein assay (35). Equal protein amounts (30 μg) were separated by 15% SDS-PAGE. The FcγRs were detected with mouse antihuman FCγRI biotin (MCA756B; 1:50), mouse antihuman FCγRII biotin (MCA1075B; 1:50) and mouse antihuman FCγRIII biotin (MCA1193B; 1:50; all Abs were from Serotec). Bands were visualized using chemioluminescence luminal reagent (sc-2048; Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to protocol. The beta (β)-actin protein level was determined by Western blot and used as a protein loading control. For RT-PCR analysis total cellular RNA was extracted from A-253 cells using the RNAzol bee isolation reagent (Tel-Test, Friendswood, TX, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg total cell RNA using the Superscript II reverse transcriptase kit (Life Technologies, NY, USA). To analyse the three different forms of FcRs, the following primer pairs were used for amplification: FCγRI, forward: 5′-AATGGC ACCTACCATTGCTC-3′ and reverse: 5′-TGTCACA GATGCATTCAGCA-3′; FCγRII, forward: 5′-TCATT GTGGCTGTGGTCATT-3′ and reverse: 5′-CCTGGG GTTCAGAGTCATGT-3′; FCγRIII, forward: 5′-CCT CAATGGTACAGGGTGCT-3′ and reverse: 5′-TGGC AGCGTCAATGAAGTAG-3′. Primers used to amplify GADPH were: GADPH forward: 5′-CAACGGAT

TTGGTCGTATT-3′ and GADPH reverse: 5′-GATG GCAACAATATCCACTT-3′ primer pairs. Each PCR was conducted in a total volume of 50 μl with 2 μl of cDNA, 50 pmol of each primer, 0.25 mM of each dNTP, 2 mM $MgCl_2$, 2 U AmpliTaq DNA polymerase and 1X PCR buffer (Roche Molecular Systems, Basel, Switzerland). The following cycles were performed: an initial denaturing step of 4 min at 95°C, followed by 45 cycles with denaturation for 1 min at 95°C, annealing for 1 min at 56°C (GADPH), 46°C (FCγRI), 47°C (FCγRII), 48°C (FCγRIII), elongation for 1 min at 72°C and a final extension for 7 min at 72°C. The expected size of PCR products is 430 bp for GADPH, 111 bp for FCγRI, 230 bp for FCγRII, 145 bp for FCγRIII. PCR products were visualized with ethidium bromide in 1.2% (w/v) agarose gel electrophoresis and confirmed by sequencing. The monocytic cell line U-937 was used as control for the entire experimental procedure.

Uptake of anti-Ro and anti-La autoantibodies by A-253 cells

The internalization of anti-Ro and anti-La autoantibodies was studied by fluorescence microscopy. The A-253 cells (1×10^6 cells/well) were distributed in well microculture plates (Nunc) after placement of a 175 μm-cellocate glass (Eppendorf). The cells were incubated with anti-Ro and anti-La (20 μg/ml) at 37°C for 2 h, fixed with 2% (w/v) paraformaldehyde in PBS for 10 min, and permeabilized with 0.1% (v/v) Triton X-100 (Bio-Rad, Hercules, CA, USA) in PBS for 5 min. The fixed cells were incubated with goat antihuman IgG-FITC antibody (sc-2456; 1:200; from Santa Cruz Biotechnology, CA, USA) at room temperature for 40 min. Intracellular autoantibodies were observed under a fluorescence microscope. To confirm the internalization of autoantibodies, cells were pre-treated for 2 h with cytochalasin D (Sigma; 15 μg/ml, w/v), an agent disrupting cytoskeletal filaments, and then treated with anti-Ro and anti-La for 2 h at 37°C. To examine the role of Fc receptors in anti-Ro and anti-La internalization, cells were treated with Fc fragments from human IgG (Chemicon) at a concentration of 200 μg/ml (v/v) for 2 h at 37°C, to block FcγRs. At the end of the incubation period, anti-Ro and anti-La autoantibodies (20 μg/ml) from Sjögren IgG were added and the cells were incubated for 2 h at 37°C. The internalization assay was conduced as above. Healthy IgG were used as control. To identify the class of FcγRs involved in the penetration A-253 cells were pre-treated with mouse monoclonal antibody (mAb) antihuman FCγRI (sc-1184), mouse mAb antihuman FCγRII (sc-20054) and mouse mAb antihuman FCγRIII (sc-19620; all purchased from Santa Cruz Biotechnology) for 2 h at 37°C to block internalization of anti-Ro and anti-La autoantibodies.

A-253 cell treatment for detection of apoptosis

To evaluate apoptosis, cell cultures were subjected to different treatments: (i) treatment with anti-Ro and anti-La autoantibodies (20 μg/ml) from Sjögren IgG for 48 h; (ii) treatment with human antibody against Ro/SS-A (20 μg/ml) and human antibody against

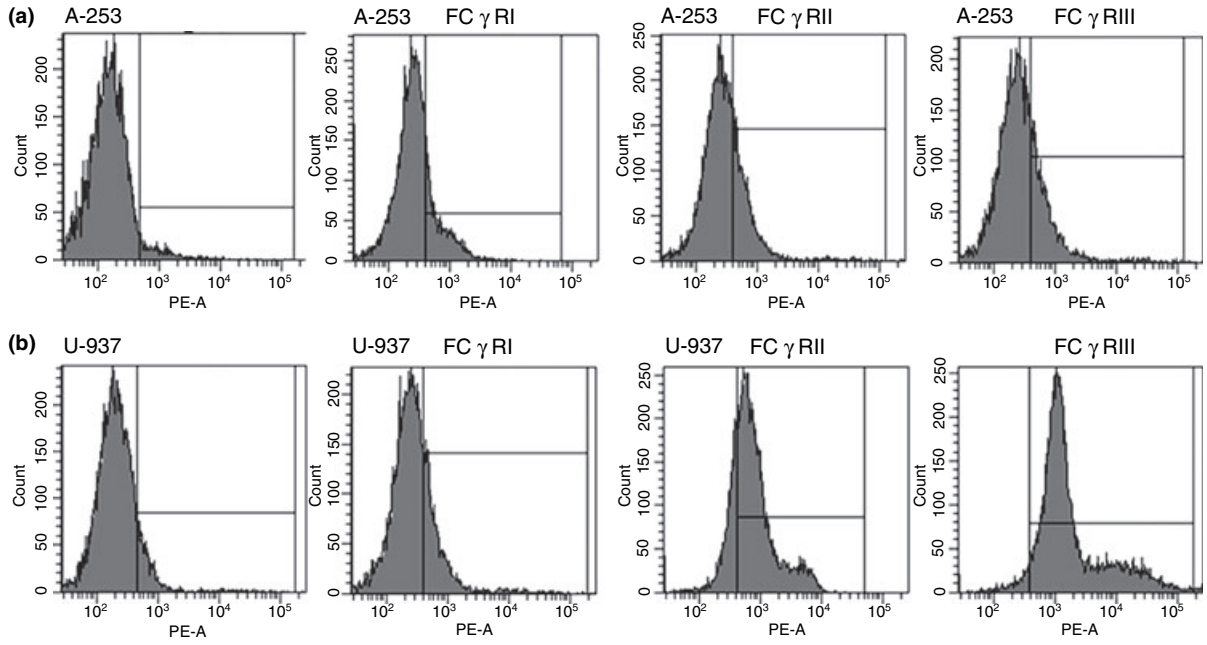

Figure 1 Flow cytometric analysis of FCγRI, FCγRII and FCγRIII receptors expression on A-253 cell membrane. Example of flow cytometric images from one representative experiment. FCγRI, FCγRII and FCγRIII receptors expression was assessed with mAbs mouse anti-human FCγRI biotin, mouse anti-human FCγRII biotin and mouse anti-human FCγRIII biotin. Streptavidin-RPE was used for secondary detection. Panel (a) represents the percentage of expression of FCγRI (40%), FCγRII (44%) and FCγRIII (46%) on A-253 cell membrane; panel (b) represents the expression of FCγRI (50%), FCγRII (80%) and FCγRIII (98%) on U-937 control cell membrane (mean ± SE of five independent experiments).

La/SS-B (20 μg/ml), sodiumazide free, for 48 h; (iii) treatment with healthy IgG fractions (20 μg/ml) for 48 h; (iv) treatment with $F(ab')_2$ fragments (20 μg/ml) from anti-Ro and anti-La autoantibodies for 48 h and (v) pre-treatment for 2 h with exogenous Fc fragments from human IgG at a concentration of 200 μg/ml before incubation with anti-Ro and anti-La autoantibodies (20 μg/ml) from Sjögren IgG. Controls included both untreated cells and cells incubated for 48 h with 5 μg/ml of the pro-apoptotic agent actinomycin D (Sigma).

Concentration of anti-Ro and anti-La autoantibodies used in apoptosis assays

To determine the optimum concentration of anti-Ro and anti-La autoantibodies from Sjögren IgG to employ in the experimental procedure, the Vibrant Apoptosis Assay kit (Molecular Probes, Inc., Eugene, OR, USA) was used as previously described in detail (18). Apoptotic cells appear green-stained, necrotic cells appear red-stained, viable cells show little or no fluorescence. A-253 cells were treated with growing concentrations of anti-Ro and anti-La autoantibodies from Sjögren IgG (5, 10 and 20 μg/ml) for 48 h. 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.

Assay for DNA fragmentation in A-253 cell line

Degradation of the A-253 cell line DNA was used as an index of apoptosis. 10^7 A-253 cells variously treated 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., Cincinnati, OH, USA) was added to cells in the presence of proteinase K (Sigma; 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 (Sigma; 20 μg/ml) was added for 1 h at 37°C, in order to eliminate contaminating RNA. The DNA extract 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 (50 μg for well) in a 2% (w/v) agarose gel and visualized by ethidium bromide staining.

Apoptosis inhibition using exogenous Fc fragment of IgG

To support the hypothesis that anti-Ro and anti-La autoantibodies have to penetrate via Fcγ receptors to trigger apoptosis, the A-253 cells (1×10^6 cells/well) were incubated with Fc fragments from human IgG at a concentration of 200 μg/ml (v/v) for 2 h at 37°C. At the end of incubation period, anti-Ro and anti-La autoantibodies (20 μg/ml) from Sjögren IgG were added and the cells were incubated for 48 h at 37°C. The inhibition

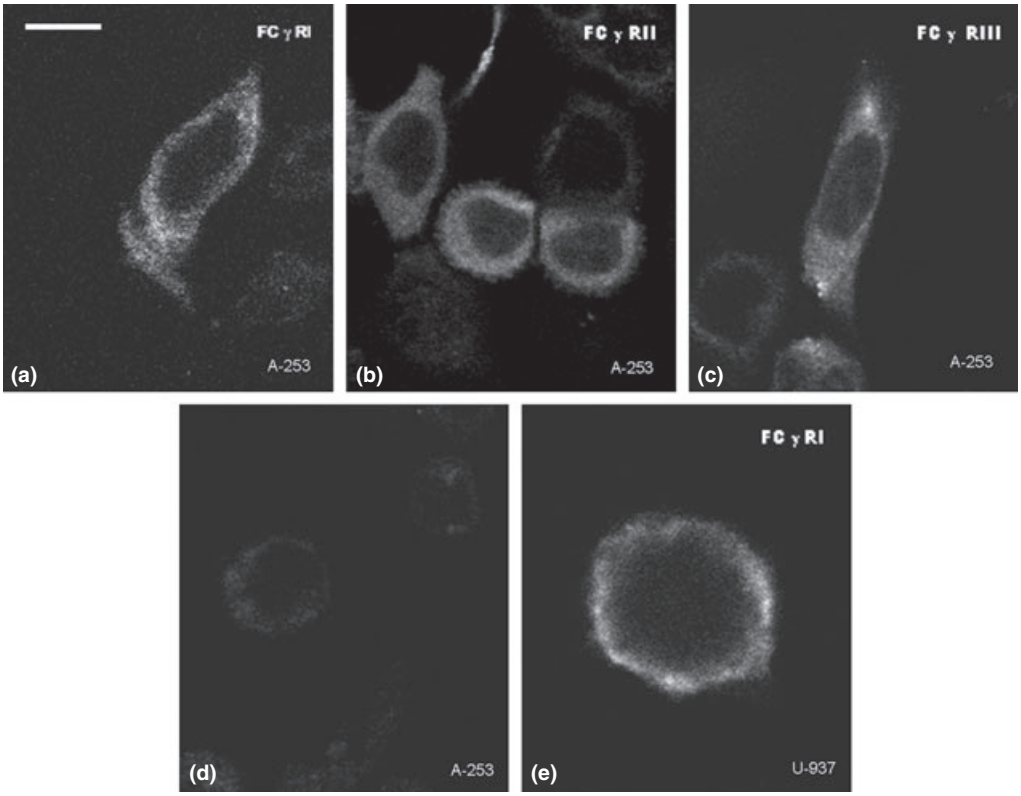

Figure 2 Confocal microscopy of FCγRI, FCγRII and FCγRIII receptors expression in A-253 cells. Cells were treated with biotinylated anti-human FCγRI, biotinylated anti-human FCγRII and biotinylated anti-human FCγRIII. Streptavidin (FITC) was used for FITC secondary detection. Expression of FCγRI (a), FCγRII (b) and FCγRIII (c) on the A-253 cell membrane; untreated control cells (d); FCγRI expression on the U-937 cell membrane (e). Data on FCγRII, FCγRIII expression on the U-937 cell membrane not shown; scale bar = 10 μm.

of apoptosis by Fc fragments was evaluated in terms of percentage of apoptotic cells, using the Vibrant Apoptosis Assay kit, and in terms of DNA fragmentation using the total cellular DNA, extracted as specified in Section: Assay for DNA fragmentation in A-253 cell line, separated by electrophoresis in a 2% (w/v) agarose gel and visualized by ethidium bromide staining.

Western blot analysis of caspases activation

The total cell content of active caspase-3, -8, -9 and of caspase-3 substrate PARP was analysed by Western blot. A-253 cells, treated as in the A-253 cells treatment Section, were washed twice, detached with ice-cold PBS, collected and centrifuged at 600 *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, 0.2 U/ml aprotinin] for 30 min on ice. After incubation, the obtained lysate was vortexed and then centrifuged at 12 800 *g* for 10 min; the protein concentration in the supernatant was spectrophotometrically determined by Bradford's protein assay (35), and the lysate was subjected to SDS-PAGE (30 μg for well) and nitrocellulose membrane transfer. After the transfer, 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 1X (T-PBS). Membranes were then incubated for 90 min with goat anticaspase-3 pAb (sc-1225; 1:500), rabbit anti-PARP pAb (sc-7150; 1:200), rabbit anticaspase-9 pAb (sc-8355; 1:200), rabbit anticaspase-8 pAb (sc-7890; 1:500; all pAbs from Santa Cruz Biotechnology). Bound Ab were visualized with bovine antirabbit IgG-HRP (sc-2370; 1:5000; Santa Cruz Biotechnology) or rabbit antigoat IgG-HRP (sc-2768; 1:5000; Santa Cruz Biotechnology). Bands were visualized using chemioluminescence luminal reagent. The β-actin protein level was determined by Western blot and used as a protein loading control.

Statistical analysis

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

Results

Evidence for FCγ RI, FCγ RII and FCγ RIII expression in A-253 cells

FACS analysis, confocal microscopy, Western blot and RT-PCR were adopted to visualize the FCγRI, FCγRII and FCγRIII receptors on the A-253 cell membrane. Fig. 1, related to flow cytometric analysis, demonstrate the expression of FCγRI (40%), FCγRII (44%) and

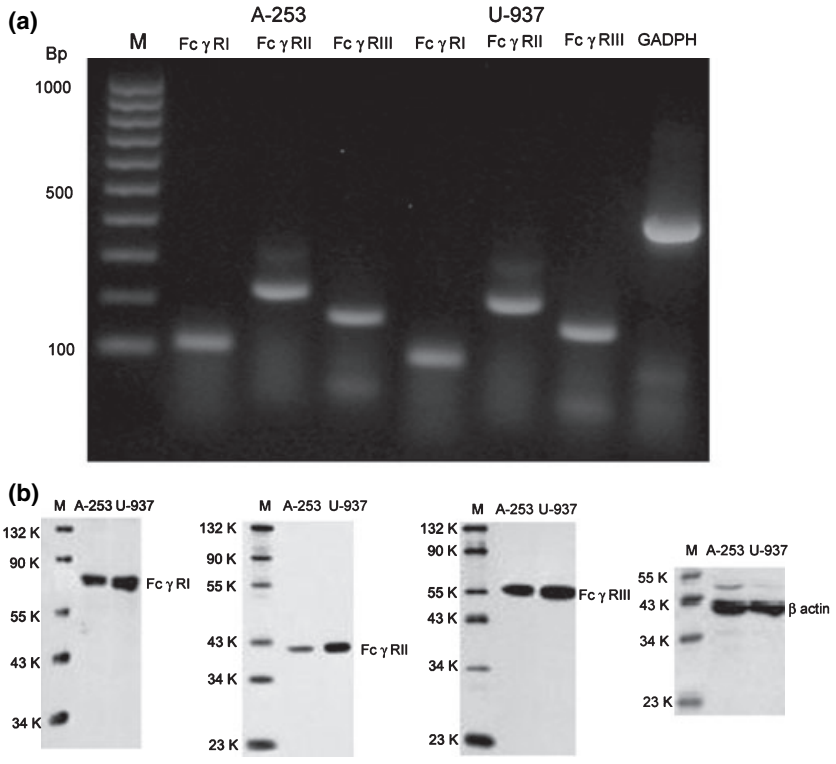

Figure 3 RT-PCR and Western blot analysis of FCγRI, FCγRII and FCγRIII receptors in A-253 cells. RT-PCR products of the expected size were visualized through agarose gel electrophoresis. M, marker. RT-PCR of GADPH was used as control (a). Western blot was carried out using membrane protein extract of A-253 cells. Bands visualized correspond to FCγRI (75 kDa), FCγRII (40 kDa) and FCγRIII (55 kDa) (b).

FCγRIII (46%) on the A-253 cell membrane (panel a). These results were confirmed by immunofluorescence assay (Fig. 2) that evidences the expression of the members of the FcγRs on the A-253 cell membrane (a = FCγRI; b = FCγRII; c = FCγRIII). No fluorescence was detected in cells treated only with paraformaldehyde or with streptavidin (FITC) (data not shown). Western blot, carried out using membrane protein extract of A-253 cells, visualizing the bands corresponding to FCγRI (75 kDa), FCγRII (40 kDa) and FCγRIII (55 kDa) (Fig. 3b), and RT-PCR, that showed clear expression of FCγRI, FCγRII and FCγRIII mRNA, proved the expression of FCγRI, FCγRII and FCγRIII on the A-253 cell membrane (Fig. 3a).

Anti-Ro and anti-La autoantibodies concentration

The nuclear morphology of A-253 cells, treated with growing concentrations of anti-Ro and anti-La autoantibodies from Sjögren IgG, was analysed by the Vybrant Apoptosis Assay Kit (Molecular Probes Inc., Eugene, OR, USA). The percentage of apoptotic A-253 cells at different concentrations of anti-Ro and anti-La IgG is illustrated in Fig. 4. When anti-Ro IgG was used at concentration of 20 μg⁄ml the percentage of apoptotic cells was 79 ± 1.2. At the same concentration of anti-La IgG, the percentage of apoptosis was 75 ± 2.7. The concentration of 20 μg⁄ml resulted optimum and was employed in all experimental procedures. Healthy IgG at the same concentration had no effect on the apoptotic phenomenon.

Uptake of anti-Ro and anti-La autoantibodies by A-253 cells

To examine the uptake of anti-Ro and anti-La autoantibodies purified from Sjögren IgG by A-253 cells an immunofluorescence assay was performed using goat anti-human IgG-FITC antibody (Fig. 5). The cells treated with anti-Ro (Fig. 5a) and anti-La (Fig. 5b) autoantibodies showed higher fluorescence, located in the cytoplasm or inside the nucleus, than control cells treated with healthy IgG (Fig. 5c), that showed a low fluorescence signal homogeneously distributed inside the cells. Pre-treatment with

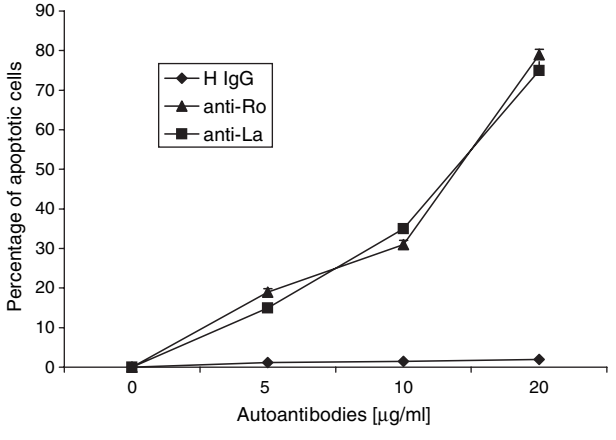

Figure 4 Percentage of apoptotic cells at growing concentrations of anti-Ro and anti-La autoantibodies from Sjögren IgG. The percentage of apoptotic cells was assessed using the YO-PRO-1 assay kit. The A-253 cells treated with growing concentrations of anti-Ro (▲), anti-La (■) and healthy IgG (♦) were observed by fluorescence microscopy (mean ± SE of five independent experiments).

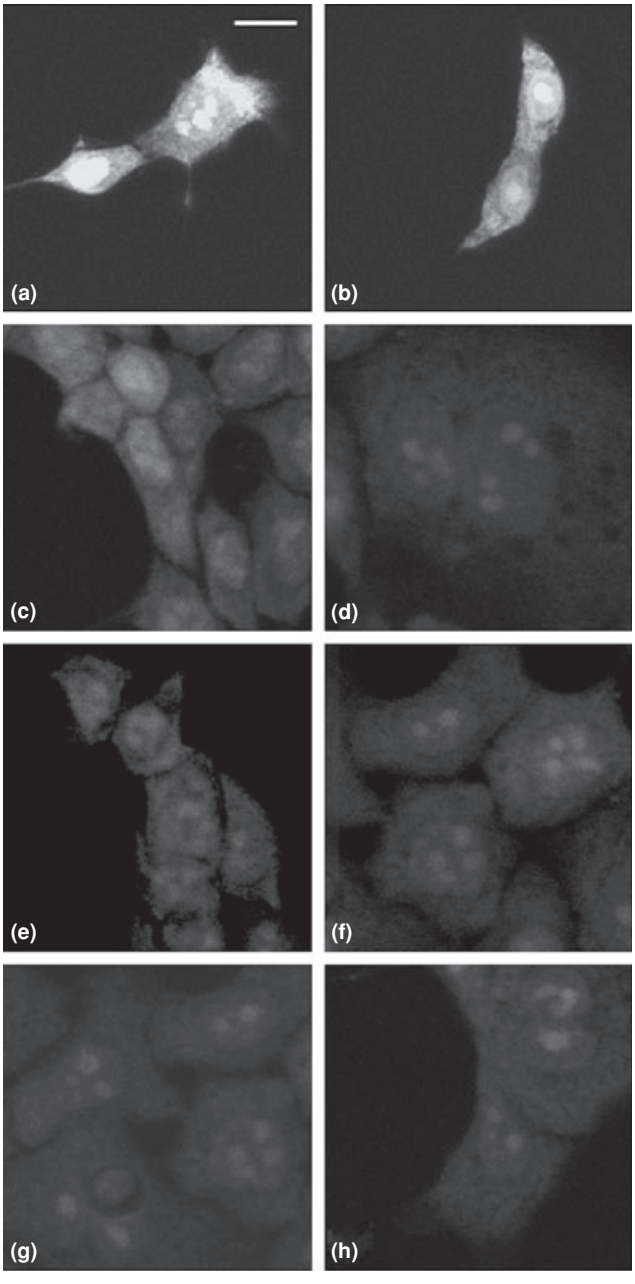

Figure 5 Internalization of anti-Ro and anti-La autoantibodies from Sjögren IgG by A-253 cells, detected by fluorescence microscopy. The anti-Ro and anti-La internalization was revealed using goat anti-human IgG-FITC antibody. Cells treated with anti-Ro (a); cells treated with anti-La (b); cells treated with healthy IgG (c); cells treated with goat anti-human IgG-FITC antibody (d); cells pre-treated with cytochalasin D and anti-Ro (e); cells pre-treated with cytochalasin D and anti-La (f); cells pre-treated with Fc fragments of IgG and anti-Ro (g); cells pre-treated with Fc fragments of IgG and anti-La (h). Scale bar = 10 μm.

cytochalasin D blocked the anti-Ro (Fig. 5e) and the anti-La (Fig. 5f) internalization. Cells treated with the goat anti-human IgG-FITC antibody alone did not show any fluorescence (Fig. 5d), while cells treated without permeabilization buffer prior to the incubation of the secondary antibody showed positive membrane labelling (data not shown). To demonstrate that anti-Ro and anti-La autoantibodies enter via the FcγRs in A-253 cells, the cells were pre-treated with the Fc fragment of IgG or with mAbs specific for FcγRs. We observed that the exogenous Fc fragment blocks anti-Ro and anti-La Abs internalization (Fig. 5g,h). In addition, as showed by block exerted by mAbs to FcγRs, both FcγRI and FcγRII and FcγRIII are involved in anti-Ro and anti-La autoantibodies penetration (Fig. 6).

Exogenous Fc fragment of IgG blocks apoptosis induced by anti-Ro and anti-La

The treatment with Fc fragments before the addition of anti-Ro and anti-La autoantibodies inhibits apoptosis in A-253 cells. When the A-253 cells were treated with anti-Ro and anti-La autoantibodies the percentage of apoptotic cells was 77 ± 1.1 and 75 ± 1.9, respectively, while pre-treatment with Fc fragments from human IgG significantly ($P < 0.05$) decreased the percentage of apoptosis induced by anti-Ro and anti-La (21 ± 2.5 and 20 ± 2.5, respectively; Fig. 7). DNA fragmentation caused by anti-Ro and anti-La autoantibodies from SS IgG was blocked by pre-treatment with the Fc fragments (Fig. 8). Figure 8 shows DNA fragmentation in cells treated with the pro-apoptotic agent actinomycin D and with the human anti-Ro/SS-A and human anti-La/SS-B. No apoptotic effect was observed in cells treated with $F(ab')_2$ fragments from anti-Ro and anti-La autoantibodies and treated with IgG purified from healthy donors.

Anti-Ro and anti-La from Sjögren IgG active intrinsic and extrinsic apoptotic pathways

Cleavage of the initiator caspases-8 and -9 and of the effector caspase-3 and active caspase-3 substrate, PARP, was analysed by Western blot. Caspases activation was evaluated in A-253 cells treated with actinomycin D, IgG purified from healthy sera, anti-Ro autoantibodies from Sjögren IgG, anti-La autoantibodies from Sjögren IgG, $F(ab')_2$ fragments from anti-Ro and anti-La autoantibodies, Fc fragments from human IgG plus anti-Ro, Fc fragments plus anti-La and in untreated control cells. Cleaved caspase-3 was detected in actinomycin D-treated cells as well in anti-Ro and anti-La Abs treated cells. Only the caspase-3 precursor was detectable in cells treated with Fc fragments of IgG plus anti-Ro and anti-La antibodies, as well as in cells treated with $F(ab')_2$ fragments from anti-Ro and anti-La autoantibodies (data not shown) and H IgG (Fig. 9, panel a). Caspase-3 activation was confirmed by analysis of PARP cleavage; treatment of A-253 cells with anti-Ro and anti-La autoantibodies induced cleavage of the full-length PARP into the 89 kDa subunit. No cleavage of the PARP was observed in A-253 control cells, in cells treated with $F(ab')_2$ fragments from anti-Ro and anti-La autoantibodies (data not shown) and in cells treated with IgG from healthy donors, and pre-treatment with Fc fragments before the addition of anti-Ro and anti-La Abs inhibited PARP cleavage (Fig. 9, panel b). Anti-Ro and anti-La autoantibodies treatment induced caspase-8 precursor and caspase-9 precursor activation in A-253 cells. No proteolytic cleavage of the initiator caspases was observed indeed following pre-treatment of A-253

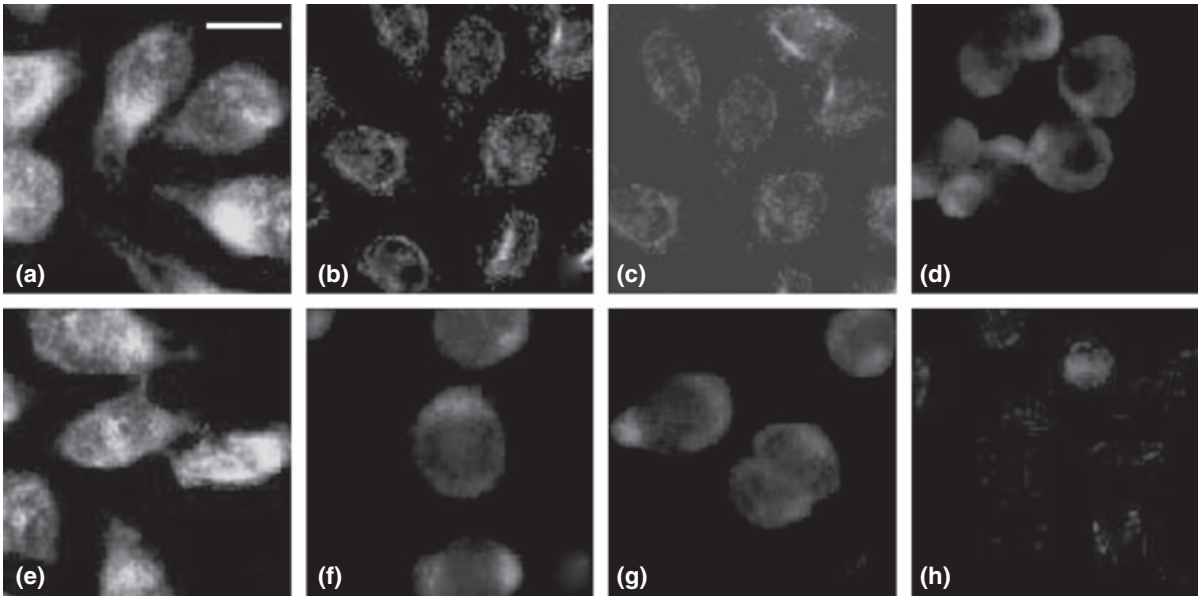

Figure 6 Block of anti-Ro and anti-La internalization through mAbs specific for FcγRs visualized by fluorescence microscopy. A-253 cells treated with anti-Ro autoantibodies (a); cells pre-treated with mAb specific for FCγRI (b), FCγRII (c) and FCγRIII (d) before the addition of anti-Ro autoantibodies. A-253 cells treated with anti-La autoantibodies (e); cells pre-treated with mAb specific for FCγRI (f), FCγRII (g) and FCγRIII (h) before the addition of anti-La autoantibodies. Scale bar = 10 μm.

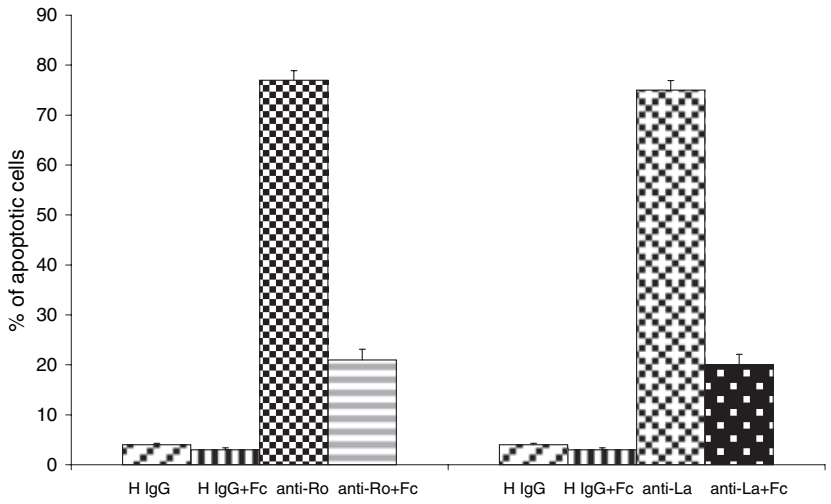

Figure 7 Pre-treatment of A-253 cells with Fc fragments from human IgG decreases the percentage of apoptosis induced by anti-Ro and anti-La autoantibodies. The percentage of apoptotic cells was assessed using the YO-PRO-1 assay kit. Cells submitted to different treatments were observed under fluorescence microscopy. H IgG, cells treated with IgG purified from healthy sera; H IgG + Fc, cells pre-treated with Fc from human IgG and with IgG purified from healthy sera; anti-Ro, cells treated with anti-Ro autoantibodies; anti-Ro + Fc, cells pre-treated with Fc from human IgG and with anti-Ro; anti-La, cells treated with anti-La autoantibodies; anti-La + Fc, cells pre-treated with Fc from human IgG and with anti-La (mean ± SE of five independent experiments).

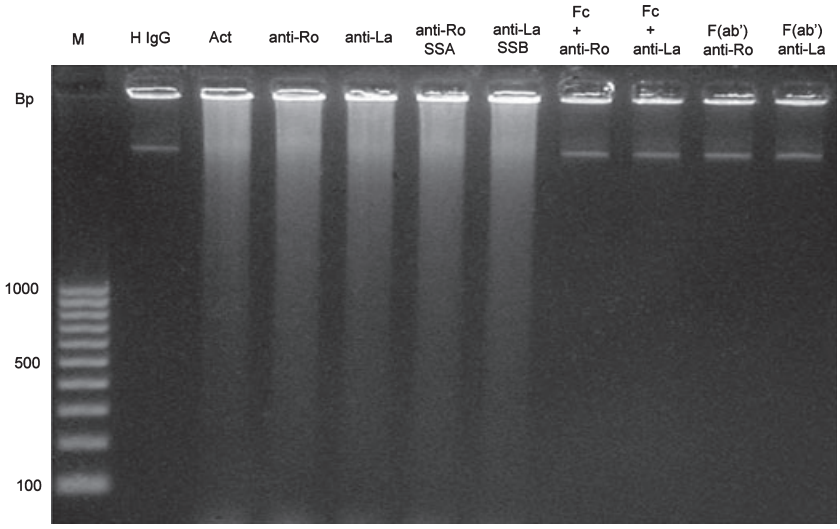

Figure 8 Anti-Ro and anti-La autoantibodies induce DNA laddering in A-253 cells. The cells were treated with healthy IgG (lane H IgG), anti-Ro purified from Sjögren IgG (lane anti-Ro), anti-La purified from Sjögren IgG (lane anti-La); human anti-Ro/SS-A (lane anti-Ro/SS-A); human anti-La/SS-B (lane anti-La/SS-B); Fc fragments from human IgG plus anti-Ro from Sjögren IgG (lane Fc + anti-Ro); Fc fragments from human IgG plus anti-La from Sjögren IgG (lane Fc + anti-La); $F(ab')_2$ fragments from anti-Ro autoantibodies [lane F(ab′) anti-Ro] and $F(ab')_2$ fragments from anti-La autoantibodies [lane F(ab′) anti-La]. 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.

cells with the Fc fragment of IgG before anti-Ro and anti-La were added nor in cells treated with $F(ab')_2$ fragments from anti-Ro and anti-La autoantibodies (data not shown) nor in cells treated with healthy IgG (Fig. 9, panels c and d).

Discussion

Previously published findings demonstrated that autoantibodies contained in IgG from Sjögren's syndrome patients are cytotoxic to a salivary gland cell line and induce activation of the extrinsic and intrinsic apoptotic pathways (18). The present report extends these observations and sheds light on the poorly understood aspect of the apoptotic mechanisms observed in Sjögren's syndrome. Herein, we have demonstrated that anti-Ro and anti-La autoantibodies, strongly correlated with SS and present in 70–90% of patients (38), bind and penetrate the salivary gland cell line causing cellular dysfunction through apoptosis.

Autoantibodies against the Ro and La proteins in patients with Sjögren's syndrome were described in 1975

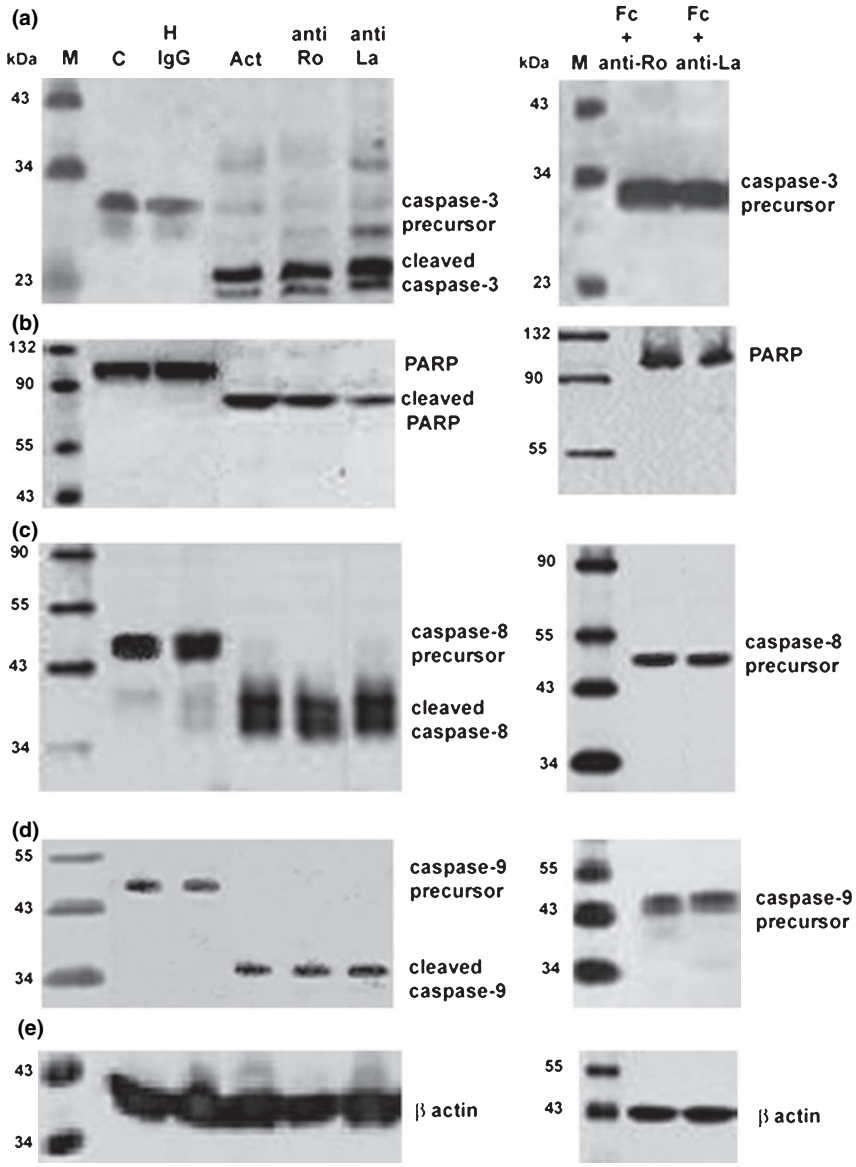

Figure 9 Proteolytic cleavage of caspases in the A-253 cell line. Blots were probed with polyclonal antibodies for caspase-3 (panel a), PARP (panel b), caspase-8 (panel c), caspase-9 (panel d) and β-actin (panel e). M, markers; C, untreated control cells; H IgG, A-253 treated with IgG purified from healthy sera; Act, A-253 cells treated with actinomycin D; anti-Ro, A-253 treated with anti-Ro purified from Sjögren IgG; anti-La, A-253 treated with anti-La purified from Sjögren IgG; Fc + anti-Ro, A-253 treated with Fc fragments from human IgG plus anti-Ro from Sjögren IgG; Fc + anti-La, A-253 treated with Fc fragments from human IgG plus anti-La from Sjögren IgG.

by Alspaugh and Tan (39). These circulating autoantibodies have since been reported to be correlated with the sicca syndrome, and the presence of anti-Ro and anti-La autoantibodies is the principal immunological criterion inserted in the European classification criteria for primary Sjögren's syndrome (1). Although there is a high correlation between the presence of serum autoantibodies, in particular of anti-Ro and anti-La, and the development of SS, it is still unclear why cellular components, such as Ro and La proteins, that participate in important biosynthetic and structural functions, are targeted for autoimmune reactions; moreover, exact role of these antibodies in the pathogenesis of the disease is uncertain. The potential pathological significance of the presence of anti-Ro and anti-La in the serum is evident in autoimmune-associated congenital heart block (40) and in neonatal lupus syndromes (41) but a convincing molecular scenario in which anti-Ro and anti-La Abs contribute to the pathogenesis of Sjögren's syndrome has yet to be described. In this report, we demonstrate that anti-Ro and anti-La autoantibodies purified from Sjögren IgG are directly pathogenic in our experimental model, consisting of the human salivary gland cell line A-253, and are able to trigger cell death through apoptotic mechanisms. Characteristic DNA laddering, observed in A-253 cells treated with anti-Ro and anti-La, demonstrated cellular apoptosis. Our data on the pathway of caspase activation that anti-Ro and anti-La autoantibodies are able to trigger in the salivary gland cell line are in agreement

with the observations made after submitting the cells to treatment with the Sjögren IgG (18). In fact, both the intrinsic and extrinsic pathways were activated, as both caspase-8 and caspase-9 cleavage occurred. The cleavage and activation of these caspases led to the cleavage and activation of effector caspase-3, confirmed by cleavage and inactivation of caspase-3 substrate poly (ADP-ribose) polymerase. In the same experimental condition, IgG purified from sera of healthy donors as well as the $F(ab')_2$ fragments from anti-Ro and anti-La autoantibodies had no apoptotic effect.

A better understanding of the mechanism by which anti-Ro and anti-La antibodies are taken up by A-253 cells is important to explain ongoing cell death. In the present study, we hypothesize that penetration of the autoantibodies may be necessary to initiate the apoptotic process. The ability of antibodies to penetrate live cells and locate their intracellular targets has long been the subject of debate (42, 43). However, it has now been demonstrated that antibodies can transgress the cell membrane of viable cells and react with their respective antigens in the intracellular matrix (44, 45). In our work, we demonstrate that anti-Ro and anti-La Abs are able to penetrate the salivary gland cell line. This was visualized by immunocytochemical analysis and is supported by the facts that penetration was blocked by using cytochalasin D, an agent that disrupts cytoskeletal filaments. As cytochalasin blocks actin polymerization, there was a marked decrease in antibody uptake. The mechanism through which the IgG antibodies penetrate has not been studied. One possibility is that the antibodies are transported via Fcγ receptors (46). Fcγ receptors recognize the Fc part of the IgG (47) and many of the effector functions of IgG are mediated through interactions with FcγRs (47). FcγRs exist primarily as membrane-bound forms but also as soluble molecules in blood (48). In humans, the protein family of Fcγ receptors comprises FcγRI (CD64), capable of binding to monomeric IgG, FcγRII (CD32) and FcγRIII (CD16), which bind only two polymeric IgG (49, 50). The overall structure of these receptors is similar: an extracellular portion made up of two (FcγRII and FcγRIII) or three (FcγRI) immunoglobulin-like domains, a hydrophobic transmembrane segment, and an intracytoplasmic tail (46, 51). Several lines of evidences show that FcγRI is expressed on macrophages, whereas FcγRII and FcγRIII have a widespread distribution on lymphoid and myeloid cells (51). The formation of the Fc/FcγR complex recruits these cells to sites of bound antigen, typically resulting in signalling and subsequent immune responses such as the release of inflammation mediators, B-cell activation, endocytosis, phagocytosis and cytotoxic attack (46, 52). All FcγRs bind the same region on IgG, Fc, yet with differing high (FcγRI) or low (Fcγ and FcγRIII) affinities (53, 54). The expression of Fcγ receptors on the surface of different cell types has not been closely studied. Investigation of Fcγ receptors distribution has been carried out to identify the FcγRs members on human epidermal keratinocytes (55), human and murine astrocytes (56) and rabbit liver cells (57). To date, there is no evidence of the expression of FcγRs members on the salivary gland cell membrane. In the studies reported herein, we tested the hypothesis that the entry of anti-Ro and anti-La autoantibodies into the salivary gland cell line is mediated by the Fcγ receptors. This is the first evidence of the expression of FcγRI, FcγRII and FcγRIII on the A-253 cell surface. Using FACS analysis and confocal microscopy these receptors were detected on the cell membrane of the salivary gland cell line. Western blot analysis, conducted using membrane protein extract, and RT-PCR confirmed these results. Then, it seems reasonable to suppose that anti-Ro and anti-La autoantibodies enter via the FcγRs to start the apoptotic process. The treatment of the A-253 cells with mAbs specific for FcγRI, FcγRII and FcγRIII blocks the anti-Ro and anti-La autoantibodies internalization. Furthermore, the inhibition of DNA fragmentation and activation of caspases cascade by the exogenous Fc fragment of IgG confirmed that the Fcγ receptors pathway is probably the one used to access the salivary gland cells and to induce apoptosis.

In conclusion, our studies show that anti-Ro and anti-La autoantibodies can be cytotoxic for salivary gland cells and can cause cell death by apoptosis. We propose that, when these autoantibodies gain access to their antigens into the cells, probably through the FcγRs family, salivary gland cells degeneration could begin and lead to glandular atrophy and loss of salivary production. This study has important connotations for a better understanding of the pathogenesis of Sjögren's syndrome, and provides a rationale for unravelling the pathogenic role of autoantibodies penetration.

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