Cellular Immunology 275 (2012) 33–41

Contents lists available at SciVerse ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Anti-M_3 peptide IgG from Sjögren's syndrome triggers apoptosis in A253 cells

Silvia Reina *, Leonor Sterin-Borda, Enri Borda

Pharmacology Unit, School of Dentistry, Buenos Aires University and Argentine National Research Council (CONICET), Ciudad Autónoma de Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 13 October 2011
Accepted 26 March 2012
Available online 3 April 2012

Keywords:
Apoptosis
Autoantibodies
Cholinergic system
Caspase-3
IgG
Metalloproteinase-3
Primary Sjögren's syndrome

ABSTRACT

Primary Sjögren's syndrome (pSS) is an autoimmune disease that targets salivary and lachrymal glands, characterized by anti-cholinergic autoantibodies directed against the M_3 muscarinic acetylcholine receptor (mAChR). The aim of this work was to evaluate if cholinergic autoantibodies contained in IgG purified from Sjögren sera could trigger apoptosis of A253 cell line. We also determined if caspase-3 and matrix metalloproteinase-3 (MMP-3) are involved in the induction of A253 cell death. Our results demonstrated that anti-cholinergic autoantibodies stimulate apoptosis and inositol phosphate (InsP) accumulation accompanied by caspase-3 activation and MMP-3 production. All of these effects were blunted by atropine and J104794, indicating that M_3 mAChRs are impacted by the anti-cholinergic autoantibodies. The intracellular pathway leading to autoantibody-induced biological effects involves phospholipase C (PLC), calcium/calmodulin (CaM) and extracellular calcium as demonstrated by treatment with U-73122, W-7, verapamil, BAPTA and BAPTA-AM, all of which blocked the effects of the anti-cholinergic autoantibodies. In conclusion, anti-cholinergic autoantibodies in IgG purified from pSS patient's sera mediates apoptosis of the A253 cell line in an InsP, caspase-3 and MMP-3 dependent manner.

1. Introduction

The presence of serum autoantibodies, such as those directed against nuclear antigens RNP/Sm, RNP-70 kD, dsDNA, ssDNA and against poly- and mono-nucleosomes, histone complex, cytoskeletal protein [1–6] and muscarinic acetylcholine receptors (mAChRs) in the salivary glands [7–10] are associated with Sjögren's syndrome (SS).

Studies from different laboratories [9–15] have demonstrated that antibodies reactive against the subtype M_3 mAChR may be the primary underlying cause for the loss of secretory function that leads to dry mouth. Unlike the numerous intracellular antigens that give rise to autoantibodies, the M_3 mAChR is a membrane-bound protein involved in the parasympathetic neurostimulation of exocrine cells. Additionally, studies have also shown that sera from patients with primary SS (pSS) or secondary SS (sSS) can inhibit smooth muscle contraction in isolated strips of bladder tissue, supporting the concept that cholinergic autoantibodies can interfere with the parasympathetic system [16]. SS is an autoimmune disease that targets salivary and lachrymal glands, leading to glandular atrophy characterized by keratoconjunctivitis sicca and xerostomia [11–13]. SS may occur alone, which is defined as pSS, or in association with other autoimmune disorders (rheumatoid arthritis, systemic lupus erythematosus, scleroderma), which is defined as sSS [17]. To this end, studies using synthetic peptides with an amino acid sequence homologous to the second extracellular loop of human salivary gland M_3 mAChR have proven that there is autoantibody specificity associated with disease pathogenesis [7].

Epithelial tissues are frequently impacted in the context of organ-specific and systemic autoimmune diseases. Accumulating evidence derived from the study of the physiology of various types of cells, not strictly immune cells, suggests that epithelia, and probably endothelia, are inherently active participants in innate and acquired immune defences [18–20]. Epithelial cells are thought to play an important role in the pathogenesis of pSS, as suggested by the occurrence of infiltrating lesions in various epithelial tissues [21–23]. In fact, epithelial cells appear to be crucially involved in the development of pSS autoimmune responses [23]. Also, epithelial cells participate in various aspects of inflammation [24], supporting the notion that epithelia cells actively participate in the pathogenesis of the chronic auto-aggressive lymphocytic glandular infiltrations that are characteristic of pSS.

Furthermore, the use of A253 cell lines has revealed that salivary gland epithelial cells are particularly susceptible to Fas-mediated as well as Fas-independent apoptotic death after stimulation with IFN-γ, probably via the downregulation of the apoptosis inhibitor protein c-FLIP [25]. In addition, signaling through CD40, a molecule that is expressed by salivary epithelia and is upregulated by IFN-γ [26], is also detected in epithelial cells. Altogether, these findings indicated that apoptosis of epithelial cells contributes to the destructive glandular lesions of pSS.

* Corresponding author. Address: Cátedra de Farmacología, Universidad de Buenos Aires, Marcelo T. de Alvear 2142-4 "B", 1122AAH Ciudad Autónoma de Buenos Aires, Argentina. Fax: +54 11 4963 2767.
E-mail address: slreina@yahoo.com (S. Reina).

0008-8749/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cellimm.2012.03.006

The association of apoptosis with autoantibody production is of recent interest. In fact, numerous diseases with an autoimmune etiology have been shown to display dysregulation of apoptosis [27]. Apoptosis or programmed cell death is a physiological process that assures cellular exchange; natural machinery eliminates the apoptotic material present in blebs and apoptotic bodies [28].

A number of G-protein coupled receptors can regulate apoptotic signaling [29,30]. β-Adrenergic stimulation in cardiac myocytes promotes apoptosis via a mechanism that may involve the dephosphorylation of the pro-apoptotic protein, Bcl-2, and initiate its translocation to the mitochondria [31,32]. Activation of the mAChRs can stimulate or inhibit cell growth and apoptosis, depending on prior levels of cellular activity [33]. The key role that mAChRs play in controlling cellular function rest not only in the diversity of receptor subtypes, but also in the fact that each receptor has the ability to activate a large array of interconnecting intracellular signaling pathways [34–36].

Our goal in the current study was to demonstrate that, in an experimental model using A253 cells line, anti-cholinergic autoantibodies in IgG purified from pSS sera specific for cellular membrane antigens could trigger the apoptotic process through the involvement of the activation of M_3 mAChR. The mechanism underlying this mAChR activation-induced enhancement of A253 cells apoptosis involves caspase-3, metalloproteinase-3 (MMP-3), phospholipase C (PLC) and calcium/calmodulin complex (CaM) via inositol phosphates (InsP) hydrolysis.

2. Materials and methods

2.1. A253 cell culture

A253 cells, a cell line derived 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) fetal bovine serum (FBS; SIGMA, MO, USA), 1% (v/v) antibiotic solution (100 U/mL penicillin and 100 μg/mL streptomycin) (Sigma–Aldrich, Saint Louis, MO, USA), and 2 mM L-glutamine (Sigma–Aldrich, Saint Louis, MO, USA) and incubated in a humidified 5% CO_2–95% air incubator at 37 °C [37]. The medium was replenished every 3–4 days. Confluent cells were sub-cultured by detaching the monolayer with 0.25% trypsin in serum-free phosphate buffered saline (PBS) (0.1 M, pH 7.2) containing 1.09 g anhydrous Na_2HPO_4, 0.32 g anhydrous NaH_2PO_4, 0.9 g NaCl and 100 mL distilled water. Only cells from passages 4 to 6 were used in the experiments and all experiments were carried out in serum-free medium. To optimize the assay, experiments were performed to establish the best incubation time and the appropriate number of cells needed. Human studies have been performed in accordance with the ethical standards established in the Declaration of Helsinki.

2.2. A253 cell treatment

To evaluate apoptosis, cell cultures were subjected to treatment with pilocarpine and anti-M_3 peptide IgG for 48 h. When different non-specific (1×10^{-6} M, atropine) or specific (4.5×10^{-8} M, J104794) mAChR antagonists or the enzymatic inhibitors of phospholipase C (PLC) (5×10^{-6} M, U-73122), calcium/calmodulin (CaM) (5×10^{-6} M, W-7), calcium flux (1×10^{-5} M, verapamil), calcium chelators (5×10^{-6} M BAPTA or 5×10^{-6} M BAPTA-AM) were used; they were added 20 min prior to the addition of the mAChR agonist pilocarpine and the anti-M_3 peptide IgG. Controls included both untreated and normal IgG-treated cells and cells incubated for 48 h with 1×10^{-7} M of the pro-apoptotic agent actinomycin D.

2.3. Study population

Females (age, 35–55 years) were selected from the metropolitan area of Buenos Aires. Subjects were divided into two groups: 25 patients with pSS and 25 healthy volunteers. A diagnosis of pSS followed the $\geqslant$4 criteria stated by Vitali et al. [38]. Twenty out of 25 patients (100%) displayed a positive biopsy with a score focus of 3.8 ± 0.07. Serologic tests were carried out for anti-Ro/SS-A and anti-La/SS-B antibodies, rheumatoid factor (RF) and antinuclear antibodies (ANA) as previously reported [39]. The frequency of anti-M_3 peptide IgG in pSS patients were significantly higher than those observed with total normal IgG (nIgG) (Table 1).

2.4. M_3 mAChR synthetic peptide

A 25-mer peptide K-R-T-V-P-D-N-Q-C-F-I-Q-F-L-S-N-P-A-V-T-F-G-T-A-I corresponding to the amino-acid sequence of the second extracellular loop of the human M_3 mAChR and non-specific unrelated 25-mer synthetic peptide (S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S) were synthesized using F-moc-amino acids activated using a 1-hydroxybenzotriazole/dicyclohexylcarbodiimide (HOBt/DCC) strategy with an automatic peptide synthesizer (Model 431A; Applied Biosystems, Menlo Park, CA, USA). The peptide was desalted and purified using high-performance liquid chromatography (HPLC). It was then subjected to amino-terminal sequence analysis using automatic Edman degradation (470 A Sequence; Applied Biosystems).

2.5. Purification of human immunoglobulin G (IgG)

The serum IgG fraction from 25 patients with pSS and 25 normal subjects was isolated using protein G affinity chromatography for protein A and standardized for protein G. Briefly, sera were loaded onto the protein G (Sigma–Aldrich, Saint Louis, MO, USA) affinity column equilibrated with 1 M Tris–HCl (pH 8.0). Columns were then washed with 10 volumes of the same buffer. The IgG fraction was eluted with 100 mM glycine–HCl (pH 3.0) and immediately neutralized. IgG concentrations were determined using a radial immunodiffusion assay.

2.6. Purification of anti-M_3 peptide IgG by affinity chromatography

The IgG fraction from 25 patients with pSS and 25 normal subjects was independently subjected to affinity chromatography using the synthesized peptide covalently linked to AffiGel 15 gel (Bio-Rad, Richmond, CA, USA) as described [39]. Briefly, the IgG fraction was loaded onto the affinity column equilibrated with PBS. The non-peptide fraction was first eluted with the same buffer. Specific anti-peptide antibodies were then eluted with 3 M KSCN and 1 M NaCl, followed by immediate extensive dialysis against PBS. The IgG concentration of non-anti-peptide antibodies and specific anti-muscarinic receptor peptide antibodies were determined using a radial immunodiffusion assay. Their immuno-

Table 1
Serological test carried out on pSS patients and normal individuals.

Serological test	pSS patients	Healthy individuals
ANA	19/25 (77%)	1/25 (5%)
RF	8/25 (33%)	1/25 (5%)
Anti-Ro (SS-A)	11/25 (45%)	0/25 (0%)
Anti-La (SS-B)	10/25 (41%)	0/25 (0%)
Anti-M_3 peptide IgG	22/25 (90%)	0/25 (0%)

Distribution of serum IgG from the pSS patients and healthy individuals. The results were expressed as the number of positives per the total, with percentages in parentheses.

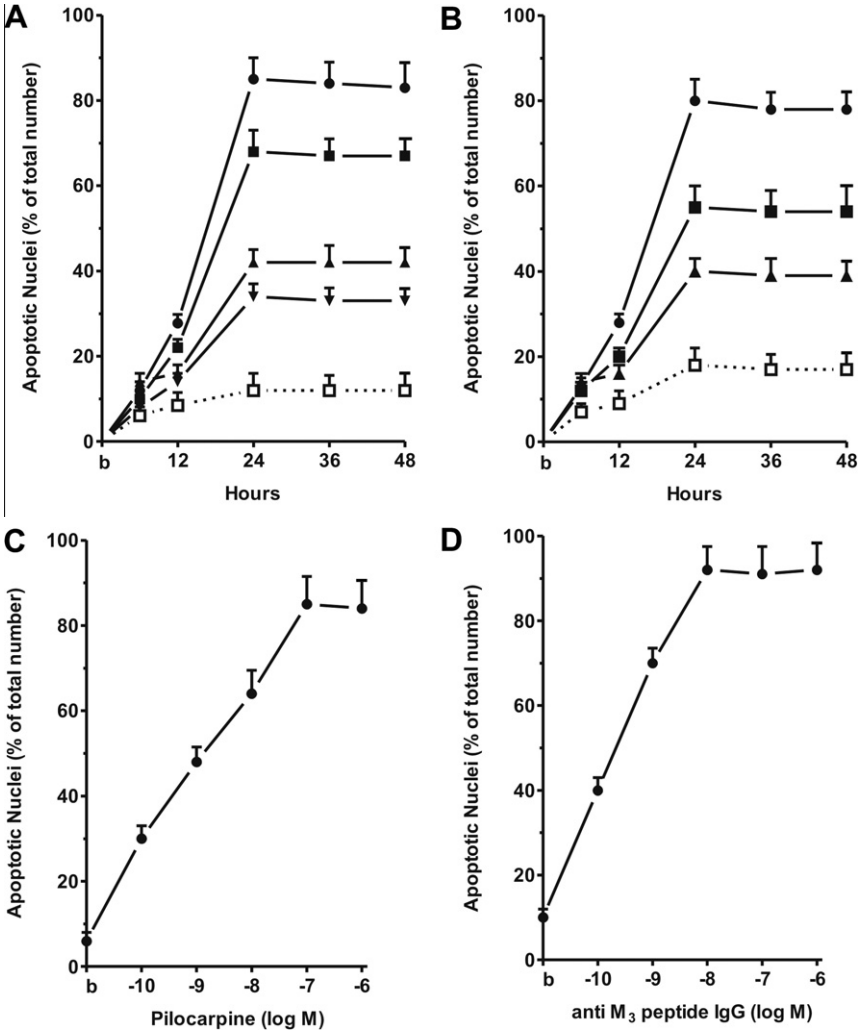


Fig. 1. Effect of pilocarpine and total IgG on apoptosis of A253 cells by TUNEL assay. (A and B) Time course of apoptotic effect of different concentrations of pilocarpine at 1×10^{-7} M (●-●), 1×10^{-8} M (■-■), 1×10^{-9} M (▲-▲), or 1×10^{-10} M (▼-▼) or total IgG at 1×10^{-7} M (●-●), 1×10^{-8} M (■-■) or 1×10^{-9} M (▲-▲). Control without drug or normal IgG (n IgG) (□–□) is also shown. Concentration–response curves for pilocarpine (C) and anti-M_3 peptide IgG (D) at 24 h of culture. Values are mean ± SEM of $n = 7$ for each group performed in duplicate.

logical reactivity against muscarinic receptor peptides was evaluated using an enzyme-linked immunosorbent assay (ELISA). The concentration of the affinity-purified anti-M_3 peptide IgG (1×10^{-7} M) that maximally increased the optical density (OD: 2.4 ± 0.2) corresponded to a total IgG concentration of 1×10^{-6} M (OD: 2.2 ± 0.2). The non-specific synthetic peptide IgG fraction eluted from the column generated OD values (0.27 ± 0.06) similar to those of normal IgG (OD: 0.25 ± 0.04). The normal IgG fraction purified using affinity column chromatography generated a negative result (OD: 0.24 ± 0.03), as did the non-specific synthetic peptide (OD: 0.23 ± 0.03).

2.7. MMP-3 assay

A253 cells (2×10^6) were cultured in McCoy's 5a modified medium (GIBCO, CA, USA) supplemented with 1% (v/v) antibiotic solution (100 U/mL penicillin and 100 μg/mL streptomycin) (Sigma–Aldrich, Saint Louis, MO, USA) and 2 mM L-glutamine (Sigma–Aldrich, Saint Louis, MO, USA) and incubated in a humidified 5% CO_2–95% air incubator at 37 °C. Pilocarpine and anti-M_3 peptide IgG were added at different times and concentrations. Blockers were added 20 min before the addition of different concentrations of pilocarpine and anti-M_3 peptide IgG. Then, the cells were homogenized into a 1.5-mL polypropylene microcentrifuge tube. For extraction of components of the extracellular matrix, the methods described by Rapraeger et al. [40] were used with minor modifications. Briefly, samples were mixed with extraction buffer (0.5 M Tris–HCl (pH 7.5), 1% Triton X-100, 10 mM $CaCl_2$ and 200 mM NaCl) at a ratio of 1:5 (weight/volume) at 4 °C and homogenized in a glass/glass conical homogenizer. The homogenate was then subject to three freezes thaw cycles of 5 min each and centrifuged at 13,000*g* for 30 min at 4 °C. The detergent-soluble supernatant was recovered and stored at −70 °C for further analyses; the insoluble pellet fractions were discarded. Protein determination was performed according to the method described by Lowry and Thiessen [41]. The enzymatic activity of MMP-3 in detergent extracts of glands was analyzed using ELISA according to the manufacturer's instructions (Amersham Matrix Metalloproteinase-3 Biotrak Activity Assay System; GE Healthcare, NJ, USA). MMP-3 production is provided as microgram per milliliter (μg/mL).

2.8. Determination of apoptosis

2.8.1. TUNEL assay

We also assayed apoptosis using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling

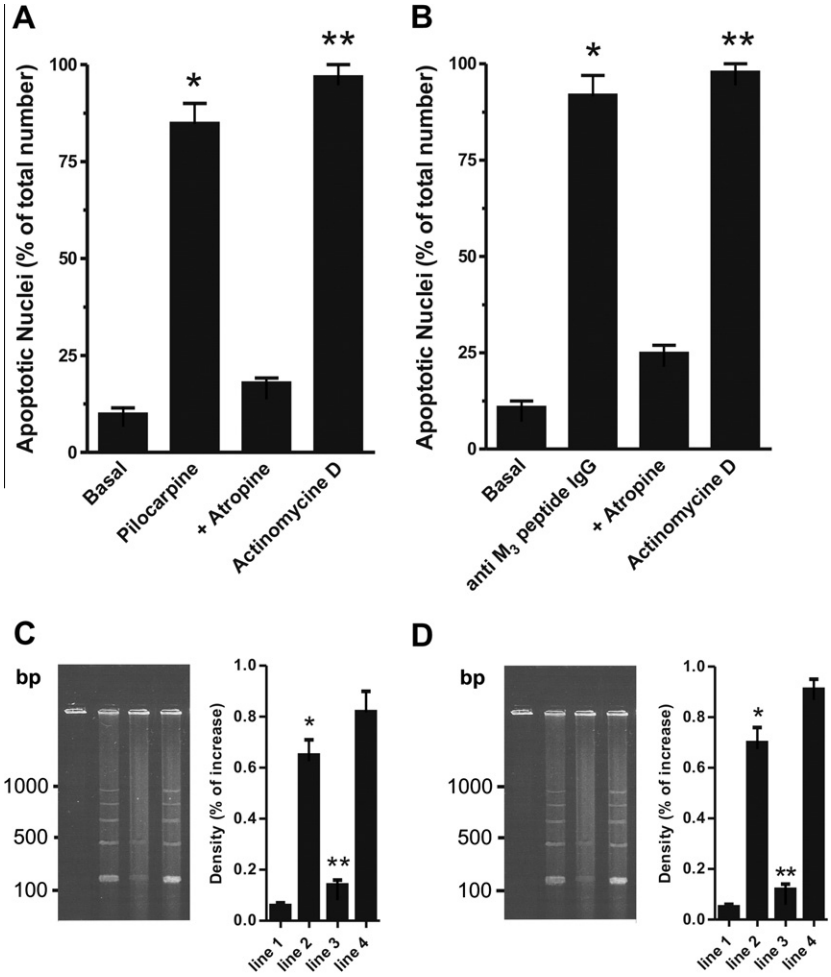


Fig. 2. Apoptotic effect measure by TUNEL of 1×10^{-7} M pilocarpine alone (A) and anti-M_3 peptide IgG alone (B) or both in the presence of 1×10^{-6} M atropine. The effect of 1×10^{-7} M actinomycin D and the basal value without pilocarpine or anti-M_3 peptide IgG are also shown. Values are mean ± SEM of $n = 5$ per group performed in duplicate. $^{*}P < 0.0001$ between pilocarpine or anti-M_3 peptide IgG alone versus atropine + pilocarpine or atropine + anti-M_3 peptide IgG or basal values. $^{**}P < 0.0001$ between actinomycin D versus basal. DNA degradation analyses of A253 cells is shown in Fig. 2 C and D. Untreated A253 cells (line 1 C and D), apoptotic effect of 1×10^{-7} M pilocarpine (line 2 C) or 1×10^{-8} M anti-M_3 peptide IgG (line 2 D) or 1×10^{-6} M atropine + pilocarpine (line 3 C) or 1×10^{-6} M atropine + anti-M_3 peptide IgG (line 3 D) and 1×10^{-7} M actinomycin D (line 4 C and D) The molecular weight standard is shown in line bp. Density values of each band beginning at 1000 bp are also shown (histograms corresponding to C and D).

(TUNEL) method, which examines DNA strand breaks that occur during apoptosis, using an in situ cell death detection reagent (Roche Molecular Biochemicals, Mannheim, Germany). We performed this assay as described previously [42]. Briefly, 2×10^6 cells were incubated in different concentrations of pilocarpine and anti-M_3 peptide IgG for different lengths of time. When the mAChR antagonist and enzymatic inhibitors were used, they were added 20 min before the pilocarpine and anti-M_3 peptide IgG was added. Then, samples were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton-X 100 in 0.1% sodium citrate. After quenching endogenous peroxidase activity with 3% (v/v) H_2O_2 and rinsing in 150 mM NaCl, 10 mM Tris (pH 7.4) buffer (TBS), terminal deoxynucleotidyl transferase (TdT) was subsequently applied for 1 h at 37 °C in a biotinylated nucleotide mix and equilibration buffer (Tris acetate, pH 7.9, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, 0.25 mM $CoCl_2$ and 24 μM biotin-dATP). The cells were then blocked with stop/wash buffer, and incubated with 100 μL streptavidin–horseradish peroxidase (HRP) (1:500 dilutions) for 30 min at room temperature. Finally, after washing several times with TBS, the samples were developed using 3,3′-diaminobenzidine (DAB) and counterstained with hematoxylin and eosin. Apoptotic nuclei (percent of total number) were evaluated at 100× magnification in 10 different fields of at least 100 cells in each field. Values are expressed as a percentage above control without drugs taken as 100%.

2.8.2. DNA fragmentation of the A253 cell line

Degradation of A253 cell DNA was used as an index of apoptosis. A253 cells (2×10^6) from each treatment were washed in PBS and lysed in 10 mM Tris (pH 7.4), 5 mM ethylenediaminetetraacetic acid (EDTA) and 1% (v/v) Triton X-100 for 20 min on ice. Then, 1 mL DNA-zol (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 using ethidium bromide staining. Band intensity was quantitated by densitometry using NIH Image software [43].

2.8.3. Caspase-3 assay

Caspase-3 activity was measured using the CaspACE Assay System Fluorometric Kit (Promega Corporation, Madison, WI, USA).

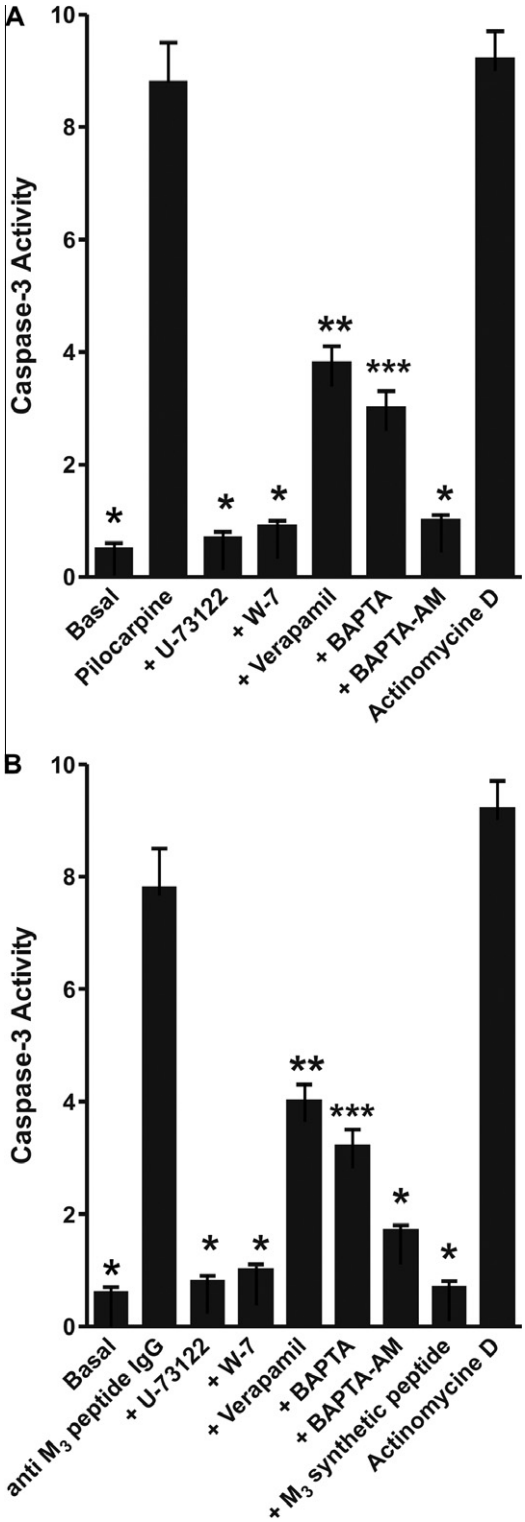


Fig. 3. The ability of 1×10^{-7} M pilocarpine alone (A), 1×10^{-8} M anti-M_3 peptide IgG alone (B), or either, in the presence of U-73122, W-7, verapamil, BAPTA, or BAPTA-AM, to induce an increase in caspase-3 activity. The effect of 1×10^{-7} M actinomycin D is also shown. Values are mean ± SEM of $n = 8$ per group performed in duplicate. $^{*}P < 0.0001$ pilocarpine alone or anti-M_3 peptide IgG alone versus basal or +U-73122, +W-7 and +BAPTA-AM. $^{**}P < 0.0008$ pilocarpine alone, anti-M_3 peptide IgG alone versus verapamil. $^{***}P < 0.0003$ pilocarpine alone, anti-M_3 peptide IgG alone versus BAPTA.

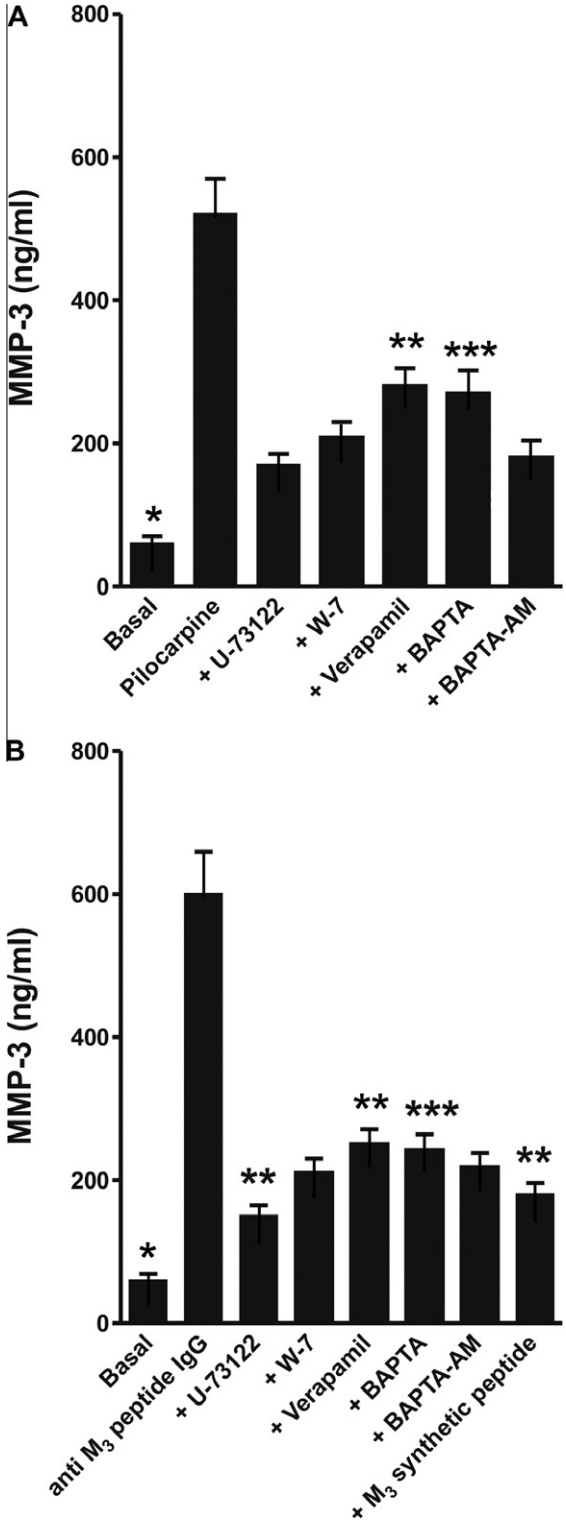
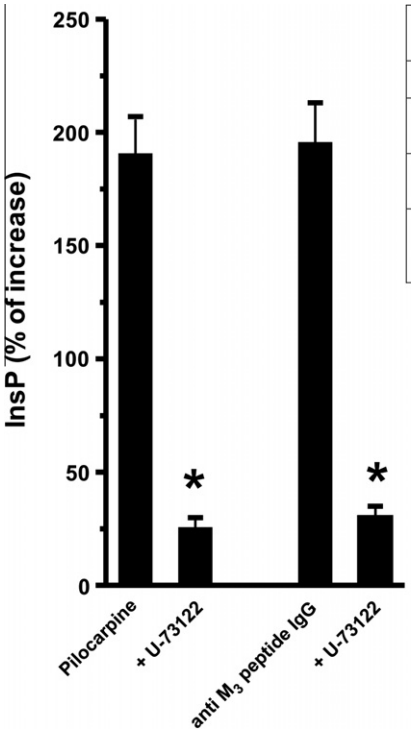


Fig. 4. The ability of 1×10^{-7} M pilocarpine alone (A), anti-M_3 peptide IgG alone 1×10^{-8} M (B) or either, in the presence of U-73122, W-7, verapamil, BAPTA, or BAPTA-AM, to promote an increase in MMP-3 activity. The basal value is also shown. Values are mean ± SEM of $n = 7$ per group performed in duplicate. $^{*}P < 0.0001$ pilocarpine alone or anti-M_3 peptide IgG alone versus basal or +U-73122. $^{**}P < 0.0003$ pilocarpine alone or anti-M_3 peptide IgG alone versus verapamil. $^{***}P < 0.002$ pilocarpine alone or anti-M_3 peptide IgG alone versus BAPTA and M_3 synthetic peptide.

Cells were initially seeded at a density of 2×10^6 in 10 cm dishes. After pilocarpine and anti-M_3 peptide IgG treatment for the indicated time, caspase-3 activity was measured by the cleavage of the fluorometric substrate Ac-DFVD-AMC according to the



Additions	Pilocarpine ($1x10^{-7}$ M)	Anti M_3 peptide IgG ($1x10^{-8}$ M)
Alone	206% ± 18	226% ± 17
Atropine ($1x10^{-6}$ M)	33% ± 5	34% ± 4
J104794 ($4.5x10^{-8}$ M)	29% ± 3	36% ± 5
M_3 synthetic peptide ($1x10^{-5}$ M)	201% ± 19	20% ± 2

Values are mean ± SEM of n = # 5 in each group performed by duplicated expressed as a percentage of increase upon basal values taken as 100%.

Fig. 5. Changes in total intracellular InsP following incubation of A253 cells in the presence of 1×10^{-7} M pilocarpine or 1×10^{-8} M anti-M_3 peptide IgG. Histogram also shows the effect of a PLC inhibitor (U-73122, 5×10^{-6} M); the Table inserts in the figure shows the effect of M_3 mAChR antagonists (atropine 1×10^{-6} M and J104794 4.5×10^{-8} M) and M_3 synthetic peptide (1×10^{-5} M). Values are mean ± SEM of $n = 6$ per group performed in duplicate and are expressed as percentage of increase upon basal value taken (basal) as 100%.

manufacturer's instructions. When enzymatic inhibitors were used, they were added 20 min before pilocarpine and anti-M_3 peptide IgG. Caspase-3 activities are the mean ± SEM of eight experiments performed in duplicated expressed in arbitrary units.

2.9. Measurement of inositol phosphates (InsP)

A253 cells (2×10^6) were incubated for 120 min in 0.5 mL Krebs–Ringer bicarbonate (KRB) gassed with 5% CO_2 in O_2 with 1 μCi [myo-^{3}H]-inositol ([^{3}H]MI) (Sp. Act. 15 Ci/mmol) from Dupont/New England Nuclear and LiCl (10 mM) was added for determination of inositol monophosphate accumulation according to the technique previously described [44]. Pilocarpine (1×10^{-7} M) and anti-M_3 peptide IgG (1×10^{-8} M) was added 30 min before the end of the incubation period and the enzymatic inhibitors and mAChR blockers were added 20 min before the addition of pilocarpine and anti-M_3 peptide IgG. Water-soluble InsP were extracted after a 120 min incubation period. Cells were washed with KRB and homogenized in 0.3 mL KRB with 10 mM LiCl and 2 mL chloroform/methanol (1:2 v/v) to stop the reaction. Then, 0.62 mL chloroform and 1 mL water were added. Samples were centrifuged at 3000g for 10 min and the aqueous phase of the supernatant (1–2 mL) was applied to a 0.7 mL column of Bio-Rad AG (Formate Form) 1×8 anion-exchange resin (100–200 mesh) suspended in 0.1 M formic acid that had been previously washed with 10 mM Tris–formic (pH 7.4). The resin was then washed with 20 volumes of 5 mM myoinositol followed by six volumes of water and InsP were eluted with 1 M ammonium formate in 0.1 M formic acid. One milliliter fractions were recovered and radioactivity was determined using scintillation counting. Peak areas were determined by triangulation [45]. Results are expressed as a percentage of the total radioactivity incorporated (1st plus 2nd peaks). In order to determine the absence of [^{3}H]MI in the eluted peaks of InsP, chromatography in silica gel 60 F254 sheets (Merck & Co, Inc., Whitehouse Station, NJ, USA) was performed using propan-2-ol-6 and NH_4 (14:5) as the developing solvent [45]. Spots were located by spraying with freshly prepared 0.1% ferric chloride in ethanol followed, after air-drying with 1% sulphosalicylic acid in ethanol. To assay radioactivity, a histogram was constructed by cutting up the sheet gel, placing each sample in Triton–toluene based scintillation fluid and counting.

2.10. Drugs

Pilocarpine, atropine, verapamil, U-73343 and actinomycin D were purchased from Sigma–Aldrich (Saint Louis, MO, USA); BAPTA, BAPTA-AM, J104094 and W-7 were purchased from Tocris Biosciences (Ellisville, MO, USA). Stock solutions were freshly prepared in the appropriate buffers. The drugs were diluted to achieve the final concentrations stated in the text.

2.11. Statistical analysis

Student's *t*-test for unpaired values was used to determine the levels of significance. When multiple comparisons were necessary, after analysis of variance, the Student–Newman–Keuls test was applied. Differences between means were considered significant if $p < 0.05$.

3. Results

Evaluation of the effects of different concentrations of pilocarpine and total IgG at varying times of culture using a TUNEL assay demonstrated that pilocarpine and total IgG increased the number of apoptotic A253 cells compared to untreated cultures or cultures treated with normal IgG (nIgG) (Fig. 1A and B). Anti-M_3 peptide

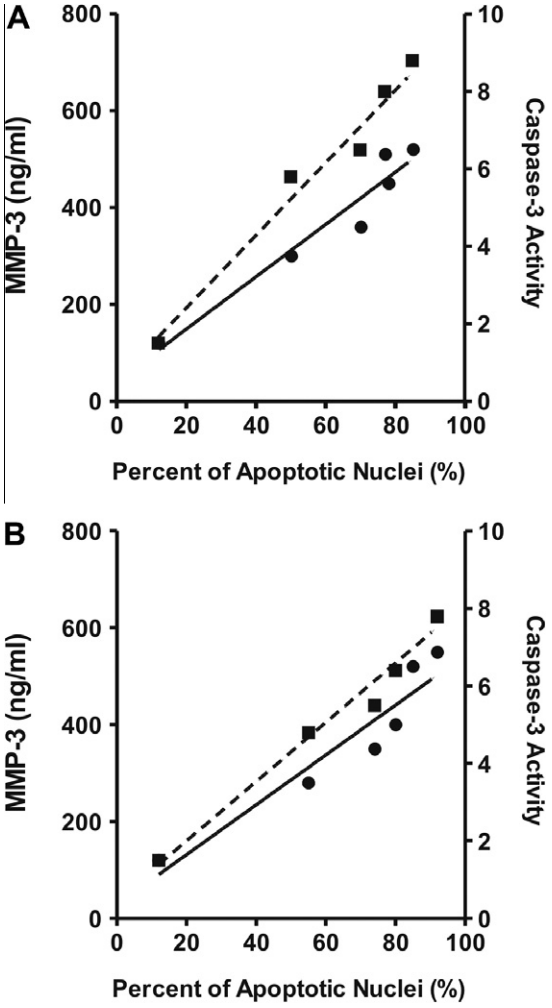


Fig. 6. Correlation in the effect of pilocarpine (A) and anti-M_3 peptide IgG (B) inducing apoptotic action and pilocarpine or anti-M_3 peptide IgG-increased MMP-3 production and caspase-3 activity. Values are mean ± SEM of $n = 5$ experiments in each group performed in duplicate.

Table 2
Influence of different compounds on caspase-3 activity and MMP-3 production in the presence of pilocarpine and anti-M_3 peptide IgG.

Additions	Caspase-3 activity	MMP-3 (ng/mL)
Basal	nc	60 ± 5.1
Pilocarpine (1×10^{-7} M)	8.81 ± 0.7	520 ± 45
+J104794 (4.5×10^{-8} M)	3.14 ± 0.2*	312 ± 30*
Anti M_3 peptide IgG (1×10^{-8} M)	7.91 ± 0.6	600 ± 50
+J104794 (4.5×10^{-8} M)	2.34 ± 0.1**	298 ± 24**
+M_3 synthetic peptide (1×10^{-5} M)	1.16 ± 0.1**	250 ± 23**
+non-specific peptide (1×10^{-5} M)	7.92 ± 0.7	589 ± 49
nIgG (1×10^{-8} M)	nc	65 ± 5.8

Results are the mean ± SEM of six experiments performed in duplicate. J104794 (M_3 specific antagonist). Control: normal IgG (nIgG).
* $P < 0.0001$ versus pilocarpine.
** $P < 0.0001$ versus anti-M_3 peptide IgG.

IgG, also, at 1×10^{-8} M, stimulated M_3 mAChR triggered a pronounced increase in the number of apoptotic A253 cells (Fig. 1D), corresponding to 1×10^{-7} M total IgG. Maximal effects of both, muscarinic agonist pilocarpine (Fig. 1C) and anti-M_3 peptide IgG (Fig. 1D) was observed 24 h using a concentration of 1×10^{-7} and 1×10^{-8} M, respectively (Fig. 1). It is important to note that the time-point taken for the concentration-dependency plots was 24 h. Also, the use of carbachol (a muscarinic agonist) gave identical results as pilocarpine and anti-M_3 peptide IgG (data not shown). No further cell death was observed if the agonist and the antibody were added a second addition due to that the steady state of the preparation was reached with the concentration cited above.

The maximal capacity 1×10^{-7} M pilocarpine and 1×10^{-8} M anti-M_3 peptide IgG to stimulate apoptosis of A253 cells was less than that induced with 1×10^{-7} M actinomycin D (Fig. 2A and B). Additionally, when 1×10^{-6} M atropine was added, 1×10^{-7} M pilocarpine and 1×10^{-8} M anti-M_3 peptide IgG displayed a reduced apoptotic capacity (Fig. 2). DNA fragmentation analyses of A253 cells treated with 1×10^{-7} M pilocarpine (line 2 C) and 1×10^{-7} M actinomycin D (line 4 C) or 1×10^{-8} M anti-M_3 peptide IgG (line 2 D) and 1×10^{-7} M actinomycin D (line 4 D) yielded the same results. Furthermore, semiquantitative analysis of the bands in each line beginning at 1000 bp, shows that pilocarpine (line 2 C) and anti-M_3 peptide IgG (line 2 D) or actinomycin D (line 4 C and D) increased significantly the intensity (Fig. 2C and D histograms). Moreover, 1×10^{-6} M atropine block the increment of density provoked by 1×10^{-7} M pilocarpine and 1×10^{-8} M anti-M_3 peptide IgG (lines 3 C and 3 D).

To confirm that cell death induced by the cholinergic agonist and anti-M_3 peptide IgG were apoptotic in nature, we evaluated the activation of caspase-3. Treatment with 1×10^{-7} M pilocarpine (Fig. 3A) or 1×10^{-8} M anti-M_3 peptide IgG (Fig. 3B) for 24 h, respectively, promoted caspase-3 activation in A253 cells. This was also observed in actinomycin D-treated cells. The incremental pilocarpine- and anti-M_3 peptide IgG-induced caspase-3 activation was blunted by additional treatment with U-73122 (5×10^{-6} M), W-7 (5×10^{-6} M), verapamil (1×10^{-5} M), BAPTA (5×10^{-6} M) or BAPTA-AM (5×10^{-6} M) (Fig. 3A and B). For all of the inhibitory agents evaluated, the concentration used had no effect on basal caspase-3 activity (data no shown).

To support that MMP-3 activation was involved in either pilocarpine- or anti-M_3 peptide IgG-induced apoptosis by M_3-mediated signaling, the MMP-3 production was also assessed in the presence of both pilocarpine (Fig. 4A) and anti-M_3 peptide IgG (Fig. 4B). Both 1×10^{-7} M pilocarpine and 1×10^{-8} M anti-M_3 peptide IgG induced an increase amount of MMP-3 (Fig. 4). Additionally, in both cases, MMP-3 production was blocked by U-73122, W-7, verapamil, BAPTA and BAPTA-AM. Furthermore, treatment with the specific M_3 synthetic peptide (1×10^{-5} M) inhibited anti-M_3 peptide IgG-mediated increase of MMP-3 production (Fig. 4B). The concentration used for each inhibitory agent had no effect on basal MMP-3 activity (data no shown).

To determine if the apoptotic effect of pilocarpine and anti-M_3 peptide IgG were related to PLC activation, we also measured InsP accumulation. Both 1×10^{-7} M pilocarpine and 1×10^{-8} M anti-M_3 peptide IgG promoted InsP accumulation (Fig. 5). Pilocarpine- and anti-M_3 peptide IgG-induced InsP accumulation was also prevented by treatment with U-73122 or the specific M_3 synthetic peptide. We also demonstrated that M_3 mAChR non-specific and specific antagonists atropine 1×10^{-6} M and J104794 4.5×10^{-8} M [46] inhibited the cholinergic agonist and anti-M_3 peptide IgG-mediated activity, respectively (Table insert in Fig. 5).

Fig. 6 demonstrates a significant correlation between the increase on apoptotic nuclei and the increase in caspase-3 activity and MMP-3 production by the action of pilocarpine (Fig. 6A) and anti-M_3 peptide IgG (Fig. 6B).

Finally, to determine if the M_3 subtype mAChR is responsible for the apoptotic effect of pilocarpine and anti-M_3 peptide IgG on A253 cells, the action of M_3 mAChR specific antagonist J104794 was studied. J104794 inhibited the stimulatory activity of pilocarpine and of anti-M_3 peptide IgG on caspase-3 and MMP-3 function (Table 2). Also, the M_3 synthetic peptide blunted the anti-M_3 peptide

IgG-induced increase of both enzymatic activities. In contrast, either the non-specific peptide or nIgG were effective in our study system at inducing MMP-3 production or caspase-3 activity.

4. Discussion

This study was undertaken to determine if cholinergic M_3 IgG autoantibodies isolated from the sera of pSS patients triggered apoptosis of A253 cell line. Our findings demonstrated that M_3 mAChR-specific antibodies induced apoptosis of A253 cells; anti-M_3 peptide IgG-mediated activation of M_3 mAChR induced apoptosis similar to pilocarpine (cholinergic agonist). Additionally, anti-M_3 peptide IgG induced apoptosis via the enhancement of InsP accumulation, which also accelerated the apoptotic process. The mechanism appears to be related to an increase in calcium influx and the anti-M_3 peptide IgG-mediated apoptosis involved increases in both caspase-3 and MMP-3 activity and M_3 mAChR-mediated caspase-3 and MMP-3 function. In contrast, anti-M_3 peptide IgG purified from the sera of healthy individuals had no effect on A253 cells.

The ability of some autoantibodies to enter viable cells and react with cellular antigens has been demonstrated by several reports [47,48]. It has been reported that polyclonal IgG anti-ribonucleoprotein isolated from autoimmune patients can penetrate live human peripheral blood mononuclear cells [49], keratinocytes [50] and epithelial cells [51]. Autoantibodies can also modify cellular functions, arrest the progression of the cell cycle and abrogate the expression of some genes [52].

Regarding the mechanism by which pilocarpine and anti-M_3 peptide IgG induces apoptosis, we found a correlation between pilocarpine- or anti-M_3 peptide IgG-induced apoptosis and pilocarpine- or anti-M_3 peptide IgG-mediated increased InsP accumulation. Thus, pilocarpine and anti-M_3 peptide IgG activation of M_3 mAChRs results in InsP accumulation, which in turn, accelerates the apoptotic process. Also, at it was previously reported pilocarpine and anti-M_3 peptide IgG are able to inhibited mucin production [53] and decreased salivary fluid production [54]. The mechanism appears to relate to an increase in intracellular calcium concentration. The initial rise in intracellular calcium may be regulated by calcium influx and by IP_3, which triggers the release of calcium from intracellular stores. The fact that inhibitors of calcium influx, PLC and CaM prevented the pilocarpine effects indicates the participation of both enzymes in pilocarpine-promoted fibroblast apoptosis. In human renal cancer cells, *m*-3M3FBS (activator of PLC) treatment induced apoptosis via PLC signaling pathways and increased the intracellular calcium level [55]. Moreover, angiotensin II stimulated cardiac fibroblast proliferation in neonatal rat cell cultures and stimulated apoptosis via the PLC pathway, which was accompanied by an increase in the intracellular calcium level [56].

In SS there is no evidence of any correlation between autoantibody penetration of salivary epithelial cells and apoptosis [57,58]. In this report, we demonstrated that, using A253 cell line, anti-M_3 peptide IgG from the sera of pSS patients is able to trigger cell death through apoptotic mechanisms. In addition, the cholinergic M_3 antibodies from the sera activate caspase-3 and MMP-3 via calcium and PLC-dependent mechanisms. Moreover, pilocarpine was able to stimulate caspase-3 and MMP-3 activity. In fact, anti-M_3 peptide IgG functions like pilocarpine, suggesting that activating M_3 mAChR are responsible for the activation of PLC and calcium signaling pathways and subsequent activation of caspase-3 and MMP-3, which stimulate apoptotic cell death. Using the same experimental conditions, the cholinergic autoantibody purified from the sera of healthy donors failed to induce apoptosis.

Altogether, these findings indicate that epithelial cells apoptosis contributes in salivary gland destructive lesions of SS, that in turn, exert a direct action of local infiltrating cytotoxic T cells and their products (cytokines and pro-inflammatory modulators) conforming an inflammation that may be involved in apoptosis [56]. The apoptotic death of epithelial cells in SS seems to involve the activation of complement and acts complementary to antibody-dependent cellular cytotoxicity by macrophages as was previously reported [58,59] and the apoptosis phenomenon is another component that play a role in the pathogenesis of SS [60]. Moreover, this may be an important step towards identifying more appropriate treatment for pSS, allowing for the development of new therapeutic strategies to prevent epithelial cell death and the consequential impairment of secretory function.

It is important to note that in salivary glands of patients with pSS the ductal epithelial and acinar cells exhibit increased apoptosis and the proliferation was mainly observed in infiltrated lymphoid cells [56]. Furthermore, the fact that the glandular environment in SS, will increase glandular pro-inflammatory substances and the presence of the M_3 muscarinic autoantibodies increasing even more these cytokines, demonstrating that the inflammatory process is a important factor(s) glandular in the cell cycle in which both proliferation and apoptosis during the course of pSS leads to atrophy and loss of function of the salivary gland in the pSS [57,58].

Finally, the present work demonstrates that anti-M_3 peptide IgG can act on A253 cells and play an important role in apoptosis, which, when combined with inflammation of the target tissue, leads to the loss of glandular function and atrophy.

5. Conclusion

The results of our studies have revealed that the activation of M_3 mAChR express in an A253 cells by pilocarpine and anti-M_3 peptide IgG are able to stimulate apoptotic cell death. The mechanisms proceed via PLC and calcium signaling pathways involving an increase in caspase-3 activity and MMP-3 production, in which, both, the extrinsic and intrinsic pathways play a role. This may be an important step towards findings some new measures for treatment of Sjögren's syndrome.

Acknowledgments

The authors thank Mrs. Elvita Vannucchi for her excellent technical assistance. This work was supported by the National Agency for Science and Technology (PICT's 02120, 01647), University of Buenos Aires (UBACYT O-017), Buenos Aires, Argentina.

References

[1] C. Vitali, S. Bombardieri, H.M. Motsopouslus, G. Balestrieri, W. Bencivelli, R.M. Bernstein, et al., Preliminary criteria for the classification of Sjögren's syndrome. Results of a prospective concerted action supported by the European Community, Arthritis Rheum. 36 (1993) 340–347.
[2] R.I. Fox, C. Robinson, J. Curd, P. Michelson, R. Bone, F.V. Howell, First international symposium on Sjögren's syndrome suggested criteria for classification, Scand. J. Rheumatol. 61 (1986) 28–30.
[3] Tapinos Ni, M. Polihronis, A.G. Tzioufas, F.N. Skopouli, Immunopathology of Sjögren's syndrome, Ann. Med. Interne (Paris) 149 (1998) 17–24.
[4] T. Martin, A.M. Knapp, S. Muller, J.L. Pasquali, Polyclonal human rheumatoid factors cross-reacting with Histone H3: characterization of an idiotope on the H3 binding site, J. Clin. Immunol. 10 (1990) 211–219.
[5] A. Ghirardello, A. Doria, S. Zampieri, R. Gerli, E. Rapizzi, P.F. Gambari, Anti-ribosomal P protein antibodies detected by immunoblot in patients with connective tissues disease: their specificity for SLE and association with IgG anticardiopolpin antibodies, Ann. Rheum. Dis. 59 (2000) 975–978.
[6] P.J. Venables, Sjögren's syndrome, Best Pract. Res. Clin. Rheumatol. 18 (2004) 313–329.
[7] S. Bacman, C. Perez Leiros, L. Sterin-Borda, O. Hubscher, R. Arana, E. Borda, Autoantibodies against lacrimal gland M_3 muscarinic acetylcholine receptors in patients with primary Sjögren's syndrome, Invest. Ophthalmol. Vis. Sci. 39 (1998) 151–156.

[8] E. Borda, L. Sterin-Borda, Autoantibodies against neonatal heart M_1 muscarinic acetylcholine receptor in children with congenital heart block, J. Autoimmun. 16 (2001) 143–150.
[9] S. Reina, L. Sterin-Borda, D. Passafaro, E. Borda, Muscarinic cholinoceptor activation by pilocarpine triggers apoptosis in human skin fibroblast cells, J. Cell. Physiol. 222 (2010) 640–647.
[10] S. Reina, B. Orman, J.M. Anaya, L. Sterin-Borda, E. Borda, Cholinoreceptor autoantibodies in Sjögren syndrome, J. Dent. Res. 86 (2007) 832–836.
[11] R.I. Fox, F.V. Howell, R.C. Bone, P. Michelson, Primary Sjogren syndrome: clinical and immunopathologic features, Semin. Arthritis Rheum. 14 (1984) 77–105.
[12] T.E. Daniels, Sjögren's syndrome, Oral Surg. Oral Med. Oral Pathol. 63 (1987) 674–675.
[13] N. Delaleu, Mv. Jonsson, R. Jonsson, Disease mechanism of Sjögren's syndrome, Drug Discov. Today 3 (2004) 329–336.
[14] H.M. Moutsopoulos, T.M. Chused, D.L. Mann, J.H. Klippel, A.S. Fauci, M.M. Frank, T.J. Lawley, M.I. Hamburger, Sjögren's syndrome (Sicca syndrome): current issues, Ann. Intern. Med. 92 (1980) 212–226.
[15] J.B. Brayer, S. Cha, H. Nagashima, U. Yasunari, A. Lindberg, S. Diggs, J. Martinez, J. Goa, M.G. Humphreys-Beher, A.B. Peck, IL-4-dependent effector phase in autoimmune exocrinopathy as defined by the NOD-IL-4-gene knockout mouse model of Sjögren's syndrome, Scand. J. Immunol. 54 (2001) 133–140.
[16] K.H. Nguyen, J. Brayer, S. Cha, S. Diggs, U. Yasunari, G. Hilal, A.B. Peck, M.G. Humphreys-Beher, Evidence for antimuscarinic acetylcholine receptor antibody-mediated secretory dysfunction in nod mice, Arthritis Rheum. 43 (2000) 2297–2306.
[17] S.A. Waterman, T.P. Gordon, M. Rischmueller, Inhibitory effects of muscarinic receptor autoantibodies on parasympathetic neurotransmission in Sjögren's syndrome, Arthritis Rheum. 43 (2000) 1647–1654.
[18] P.A. Knolle, A. Limmer, Neighborhood politics: the immunoregulatory function of organ-resident liver endothelial cells, Trends Immunol. 22 (2001) 432–437.
[19] S. Koch, K. Kohl, E. Klein, D. von Bubnoff, T. Bieber, Skin homing of Langerhans cell precursors: adhesion, chemotaxis, and migration, J. Allergy Clin. Immunol. 117 (2006) 163–168.
[20] V. Racanelli, B. Rehermann, The liver as an immunological organ, Hepatology 43 (2006) S54–S62.
[21] M.N. Manoussakis, H.M. Moutsopoulos, Sjögren's syndrome: autoimmune epithelitis, Baillieres Best Pract. Res. Clin. Rheumatol. 14 (2000) 73–95.
[22] M.N. Manoussakis, S. Boiu, P. Korkolopoulou, E.K. Kapsogeorgou, N. Kavantzas, P. Ziakas, E. Patsouris, H.M. Moutsopoulos, Rates of infiltration by macrophages and dendritic cells and expression of interleukin-18 and interleukin-12 in the chronic inflammatory lesions of Sjögren's syndrome: correlation with certain features of immune hyperactivity and factors associated with high risk of lymphoma development, Arthritis Rheum. 56 (2007) 3977–3988.
[23] H.M. Moutsopoulos, Sjögren's syndrome: autoimmune epithelitis, Clin. Immunol. Immunopathol. 72 (1994) 162–165.
[24] M.N. Manoussakis, E.K. Kapsogeorgou, The role of epithelial cells in the pathogenesis of Sjögren's syndrome, Clin. Rev. Allergy Immunol. 32 (2007) 225–230.
[25] R.F. Abu-Helu, I.D. Dimitriou, E.K. Kapsogeorgou, H.M. Moutsopoulos, M.N. Manoussakis, Induction of salivary gland epithelial cell injury in Sjogren's syndrome: in vitro assessment of T cell-derived cytokines and Fas protein expression, J. Autoimmun. 17 (2001) 141–153.
[26] I.D. Dimitriou, E.K. Kapsogeorgou, H.M. Moutsopoulos, M.N. Manoussakis, CD40 on salivary gland epithelial cells: high constitutive expression by cultured cells from Sjögren's syndrome patients indicating their intrinsic activation, Clin. Exp. Immunol. 127 (2002) 386–392.
[27] J.D. Mountz, J. Wu, J. Cheng, T. Zhou, Autoimmune disease. A problem of defective apoptosis, Arthritis Rheum. 37 (1994) 1415–1420.
[28] S. Gupta, Molecular steps of cell suicide: an insight into immune senescence, J. Clin. Immunol. 20 (2000) 229–239.
[29] L.B. Dale, M. Bhattacharya, P.H. Anborgh, B. Murdoch, M. Bhatia, S. Nakanishi, S.S.G. Ferguson, G Protein-coupled receptor kinase-mediated desensitization of metabotropic glutamate receptor 1A protects against cell death, J. Biol. Chem. 275 (2000) 38213–38220.
[30] C. Gu, Y.C. Ma, J. Benjamin, D. Littman, M.V. Chao, X.Y. Huang, Apoptotic Signaling through the beta-adrenergic Receptor a new Gs effector pathway, J. Biol. Chem. 275 (2000) 20726–20733.
[31] S. Saito, Y. Hiroi, Y. Zou, R. Aikawa, H. Toko, F. Shibasaki, Y. Yazaki, R. Nagai, I. Komuro, Beta adrenergic pathway induces apoptosis through calcineurin activation in cardiac myocytes, J. Biol. Chem. 275 (2000) 34528–34533.
[32] W.Z. Zhu, M. Zheng, W.J. Koch, R.J. Lefkowitz, B.K. Kobilka, R.P. Xiao, Dual modulation of cell survival and cell death by β_2-adrenergic signaling in adult mouse cardiac myocytes, Proc. Natl. Acad. Sci. USA 98 (2001) 1607–1612.
[33] A.A. Lanzafame, A. Christopoulos, F. Mitchelson, Cellular signaling mechanisms for muscarinic acetylcholine receptors, Receptors Channels 9 (2003) 241–260.
[34] M.J. Marinissen, J.S. Gutkind, G-protein-coupled receptors and signaling networks: emerging paradigms, Trends Pharmacol. Sci. 22 (2001) 368–376.
[35] C. Leloup, D.M. Michaelson, A. Fisher, T. Hartmann, K. Beyreuther, R. Stein, M_1 muscarinic receptors block caspase activation by phosphoinositide 3-kinase- and MAPK/ERK-independent pathways, Cell Death Differ. 7 (2000) 825–833.
[36] P. De Sarno, S.A. Shestopal, T.D. King, A. Zmijewska, L. Song, R.S. Jope, Muscarinic receptor activation protects cells from apoptotic effects of DNA damage, oxidative stress, and mitochondrial inhibition, J. Biol. Chem. 278 (2003) 11086–11093.
[37] J. Varani, R.S. Mitra, D. Gibbs, S.H. Phan, V.M. Dixit, R. Mitra Jr., T. Wang, K.J. Siebert, B.J. Nickoloff, J.J. Voorhees, All-trans retinoic acid stimulates growth and extracellular matrix production in growth-inhibited cultured human skin fibroblasts, J. Invest. Dermatol. 94 (1990) 717–723.
[38] C. Vitali, S. Bombardieri, H.M. Moutsopoulos, et al., Preliminary criteria for the classification of Sjogren's syndrome, Arthritis Rheum. 36 (1993) 340–347.
[39] S. Reina, L. Sterin-Borda, B. Orman, E. Borda, Autoantibodies against cerebral muscarinic cholinoceptors in Sjögren syndrome: functional and pathological implications, J. Neuroimmunol. 150 (2004) 107–115.
[40] A. Rapraeger, M. Jalkanen, M. Bernfield, Cell surface proteoglycan associates with the cytoskeleton at the basolateral cell surface of mouse mammary epithelial cells, J. Cell Biol. 103 (1986) 2683–2696.
[41] J.R. Lowry, R. Thiessen Jr., Studies of the nutritive impairment of proteins heated with carbohydrates; in vitro digestion studies, Arch Biochem. 25 (1950) 148–156.
[42] Y. Takada, A. Murakami, B.B. Aggarwal, Zerumbone abolishes NF-kappa B and kappaB alpha kinase activation leading to suppression of antiapoptotic and metastatic gene expression, upregulation of apoptosis, and downregulation of invasion, Oncogene 24 (2005) 6957–6969.
[43] S. Ganzinelli, T. Borda, L. Sterin-Borda, Regulation of M_1 muscarinic receptor and nNOS mRNA levels by autoantibodies from schizophrenic patients, Neuropharmacology 50 (2006) 362–371.
[44] T. Borda, A. Genaro, L. Sterin-Borda, G. Cremaschi, Involvement of endogenous nitric oxide signaling system in brain muscarinic acetylcholine receptor activation, J. Neural. Transm. 105 (1998) 193–204.
[45] M. Hokin-Neaverson, K. Sadeghian, Separation of 3H-inositol monophosphates and 3H-inositol on silica gel glass-fiber sheets, J. Chromatogr. 120 (1976) 502–505.
[46] M. Mitsuya, Y. Ogino, K. Kawakami, M. Uchiyama, T. Kimura, T. Numazawa, T. Hasegawa, N. Ohtake, K. Noguchi, T. Mase, Discovery of a muscarinic M_3 receptor antagonist with high selectivity for M_3 over M_2 receptors among 2-[(1S,3S)-3-sulfonylaminocyclopentyl]phenylacetamide derivatives, Bioorg. Med. Chem. 8 (2000) 825–832.
[47] J.M. Llerena, A. Ruiz-Arguelles, D. Alarcon-Segovia, L. Llorente, E. Diaz-Jouanen, Antibody penetration into living cells. V. Interference between two fc gamma receptor-mediated functions: antibody penetration and antibody-dependent cellular cytotoxicity, Immunology 43 (1981) 249–254.
[48] D. Alarcón-Segovia, L. Llorente, A. Ruiz-Arguelles, Antibody penetration into living cells. III. Effect of antiribonucleoprotein IgG on the cell cycle of human peripheral blood mononuclear cells, Clin. Immunol. Immunopathol. 23 (1982) 22–33.
[49] J. Ma, N. King, S.L. Chen, R. Penny, S.N. Breit, Antibody penetration of viable human cells, II: anti-RNP antibody binding to RNP antigen expressed on cell surface, which may mediate the antibody internalization, Clin. Exp. Immunol. 93 (1993) 396–404.
[50] L. Galoppin, J.H. Saurat, In vitro study of the binding of antiribonucleoprotein antibodies to the nucleus of isolated living keratinocytes, J. Invest. Dermatol. 76 (1981) 264–267.
[51] T.D. Golan, A.E. Gharavi, K.B. Elkon, Penetration of autoantibodies into living epithelial cells, J. Invest. Dermatol. 100 (1993) 316–322.
[52] D. Alarcón-Segovia, L. Llorente, A. Ruiz-Arguelles, Y. Richaud-Patin, B. Pérez Romano, Penetration of anti-DNA antibodies into mononuclear cells causes apoptosis, Arthritis Rheum. 38 (1995) S179.
[53] D. Passafaro, L. Sterin-Borda, S. Reina, E. Borda, Cholinergic autoantibodies from primary Sjögren's syndrome inhibit mucin production via phospholipase C and cyclooxygenase-2 in the rat submandibular gland, Dent. Res. J. (Isfahan) 8 (2011) 138–145.
[54] D. Passafaro, S. Reina, L. Sterin-Borda, E. Borda, Cholinergic autoantibodies from primary Sjögren's syndrome modulate submandibular gland Na+/K+-ATPase activity via prostaglandin E2 and cyclic AMP, Eur. J. Oral Sci. 118 (2010) 31–38.
[55] E.M. Jung, T.J. Lee, J.W. Park, Y.S. Bae, S.H. Kim, Y.H. Choi, T.K. Kwon, The novel phospholipase C activator, *m*-3M3FBS, induces apoptosis in tumor cells through caspase activation, down-regulation of XIAP and intracellular calcium signaling, Apoptosis 13 (2008) 133–145.
[56] R. Herrera-Esparza, J. Bollain-y-Goytia, C. Ruvalcaba, M. Ruvalcaba, D. Pacheco-Tovar, E. Avalos-Díaz, Apoptosis and cell proliferation: the paradox of salivary glands in Sjögren's disease, Acta Reumatol. Port. 33 (2008) 299–303.
[57] R. Vivar, C. Soto, M. Copaja, F. Mateluna, P. Aranguiz, J.P. Muñoz, M. Chiong, L. Garcia, A. Letelier, W.G. Thomas, S. Lavandero, G.J. Díaz-Araya, Phospholipase C/protein kinase C pathway mediates angiotensin II-dependent apoptosis in neonatal rat cardiac fibroblasts expressing AT1 receptor, J. Cardiovasc. Pharmacol. 52 (2008) 184–190.
[58] M. Sisto, S. Lisi, D. Castellana, P. Scagliusi, M. D'Amore, S. Caprio, A. Scagliusi, A. Acquafredda, M.A. Panaro, V. Mitolo, Autoantibodies from Sjögren's syndrome induce activation of both the intrinsic and extrinsic apoptotic pathways in human salivary gland cell line A253, J. Autoimmun. 27 (2006) 38–49.
[59] Tomvan Meerten, Rozemarijn S. van Rijn, Samantha Hol, Anton Hagenbeek, Saskia B. Ebeling, Complement-induced cell death by rituximab depends on cd20 expression level and acts complementary to antibody-dependent cellular cytotoxicity, Clin. Cancer Res. 12 (2006) 4027–4035.
[60] P. Manganelli, P. Fietta, Apoptosis and Sjögren syndrome, Semin. Arthritis Rheum. 33 (2003) 49–65.