

Epidermal growth factor inhibits Fas-mediated apoptosis in salivary epithelial cells of patients with primary Sjögren's syndrome

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Abstract

Objective

Cell death is detected in the ducts of labial salivary glands (LSG) of patients with primary Sjögren's syndrome (pSS). However, the counter-mechanism to inhibit the apoptotic process remains unclear. In this study, we studied the ability of epidermal growth factor (EGF) to activate the PI3K-Akt pathway and NF- κ B in primary cultured salivary gland epithelial cells (SGEC) of pSS patients.

Methods

SGEC, obtained from 2 female pSS patients, were cultured and used for Hoechst staining and deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) assay. The frequency of apoptosis, detected by Hoechst staining, was quantified, and statistical significance was determined through unpaired student's t-test.

Results

Following twelve hours of stimulation, both PI3K inhibitors and anti-Fas antibody failed to induce apoptosis in primary cultured SGEC. However, the combination of anti-Fas antibody, along with LY294002 or Bay 11-7082, induced apoptosis which was statistically more significant than apoptosis found in the control cells ($p < 0.01$). Interestingly, the apoptosis induced by anti-Fas antibody along with LY294002 was clearly inhibited by the addition of 10 ng/ml EGF. Furthermore, the results of the TUNEL assay clearly indicated apoptosis through stimulation with anti-Fas antibody and LY294002 or Bay 11-7082. Furthermore, the apoptosis was completely blocked by the addition of EGF.

Conclusion

Our results suggest that salivary epithelial cells are protected from Fas mediated apoptosis, through cell survival factors including either the PI3K-Akt pathway or NF- κ B.

Key words

Epidermal growth factor, apoptosis, Akt, NF- κ B, Sjögren's syndrome.

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Abbreviations:

EGF: epidermal growth factor
ER: estrogen receptor
IKK: IkappaB kinase
LSG: labial salivary glands
NF-κB: nuclear factor kappa B
PBS: phosphate buffered saline
PFA: paraformaldehyde
PI3K: phosphatidylinositol 3-kinase
PI: propidium iodide
pSS: primary Sjögren's syndrome
TNF: tumor necrosis factor
SGEC: salivary gland epithelial cells
TUNEL: terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling

Introduction

Primary Sjögren's syndrome (pSS) is an autoimmune disorder characterized by exocrinopathy, production of auto-antibodies, and extraglandular manifestations (1, 2). Apoptosis in the labial salivary glands (LSG) of pSS patients is also characteristic of pSS, as reported by several authors (3-7). Pro-apoptotic signals, including either Fas/Fas ligand (FasL), or perforin/granzymes, are the possible pathways for inducing apoptosis of LSG in pSS patients. However, Ohlsson *et al.* suggested that the appearance of cell death observed in the ducts of LSG was not significant (8). Bcl-2 families and X chromosome-linked inhibitor of apoptosis protein (XIAP) are reported to be molecules which can inhibit apoptosis in LSG of pSS patients (9-11). However, it is difficult to determine whether or not all the anti-apoptotic processes in LSG are entirely dependent on these systems. Under the influence of growth factors, PI3K-Akt and NF-κB are both pathways which provide resistance to apoptosis. (12-15). Recently, we showed our findings regarding the significance of both signal transductions in the pathogenesis of salivary epithelial cells in SS, indicating that both pathways were distinctly activated by stimulation by EGF (16). In this study, our focus is apoptosis which occurs in the epithelial cells of LSG of pSS patients. Furthermore, our results indicate that EGF has the potential to inhibit apoptosis through both the PI3K-Akt pathway and NF-κB.

Materials and methods

Patients

The study involved 2 female patients with pSS. Both patients fulfilled the revised criteria for the diagnosis of pSS, as proposed by the European Community (17). LSG biopsies were obtained with informed consent from all participants, and the study was conducted in accordance with the human experimental guidelines of our institution.

Anti-Fas antibody and chemical inhibitors

Anti-Fas IgM monoclonal antibody (CH-11) was purchased from MBL (Nagoya, Japan). EGF was purchased from Sigma (St. Louis, MO, USA). The

PI3K inhibitors LY294002 and wortmannin, and the Ikappa B kinase (IKK) inhibitor Bay11-7082 were purchased from Calbiochem (La Jolla, CA, USA).

Culture of primary salivary epithelial cells

Primary salivary gland epithelial cells (SGEC) culture was performed in accordance with previously investigated protocol (16). In brief, one piece of the tissue, washed in cold PBS containing 100 units/ml of penicillin and 100 mg/ml of streptomycin, was placed in a 6-well plate coated with Type I collagen (Sigma). The culture medium was a defined keratinocyte-SFM (Invitrogen Life Technologies, Carlsbad, CA, USA) with the following supplement; 0.4 µg/ml of hydrocortisone (Sigma), and 25 µg/ml of bovine pituitary extract (Kurabo, Osaka, Japan). Outgrowth of the cells was observed within one week, and after they reached confluence, they were subcultured into a 100-mm² plate coated with Type I collagen. For both Hoechst staining and TUNEL assay, the SGEC were cultured onto 12-mm² cover slips precoated with Type I collagen, Cellmatrix (Nitta Gelatin, Inc., Osaka, Japan).

Hoechst staining to quantify the frequency of apoptosis

Following growth supplement starvation for 12 hours, SGEC obtained from salivary gland biopsy were cultured onto 12-mm² cover slips precoated with Type I collagen, and were incubated for 12 hours in either the presence or absence of 50 ng/ml of EGF with either PI3K inhibitor or Bay11-7082. After incubation, the cells were fixed in PBS containing 4% PFA, immersed in methanol at -20°C for 10 min, and then rinsed in PBS. For Hoechst staining, the cells were incubated in the dark with 50 ng/ml Hoechst dye 33258 for 30 minutes at room temperature. The cells were then triple rinsed for 10 min each time in PBS, mounted in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA), and scanned using an Olympus BX50 microscope with the appropriate filter. They were then photographed using a Leica IM500 system (Wetzlar, Germany). To quantify

Competing interests: none declared.

the apoptotic cells, we counted 100 cells in 3 different fields to detect apoptotic cells. The frequency of apoptosis was reported as mean $\pm$ SD, and the mean value of each experiment was compared using unpaired student's *t*-test ($p < 0.05$; statistically significant).

Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining for detection of apoptosis

For the detection of apoptosis, we chose TUNEL staining, which can identify double-stranded DNA breaks, as previously reported (18). The SGEC were fixed in 4% PFA at 4°C for 15 minutes and then immersed in PBS with 0.5% Tween 20 and 0.2% bovine serum albumin at room temperature for 15 minutes, as per instructions of Mebstain Apoptosis kit direct (MBL, Nagoya, Japan). After a triple rinse with distilled water, they were treated with a 50 μ l terminal deoxynucleotidyltransferase (TdT) solution at 37°C for one hour. For counterstaining the nuclei, the cells were triple washed with PBS and then stained with 50 μ l of 1x propidium iodide (PI) at 4°C for 20 minutes (Trevigen, Inc., Gaithersburg, MD, USA). They were again triple washed with PBS, mounted in Vectashield mounting medium, and finally scanned and photographed by confocal microscopy (LSM5, PAS-CAL; Carl Zeiss, Jena Germany).

Results

Resistance to apoptosis of primary salivary epithelial cells stimulated with either PI3K inhibitor or IKK inhibitor

Initially, we tried to determine whether or not PI3K inhibitor alone has the potential to induce apoptosis in primary cultured SGEC. After growth factor starvation for 12 hours, the primary cultured SGEC were treated with either LY294002 or wortmannin for another 12 hours with or without stimulation of EGF. For the purpose of detecting apoptosis, we used Hoechst staining. No apoptosis was detected by addition of either PI3K inhibitor without first stimulating the cells with EGF for 12 hours (Fig. 1A). We subsequently attempted to stimulate the epithelial cells with these PI3K inhibitors up to 48 hours. Again,

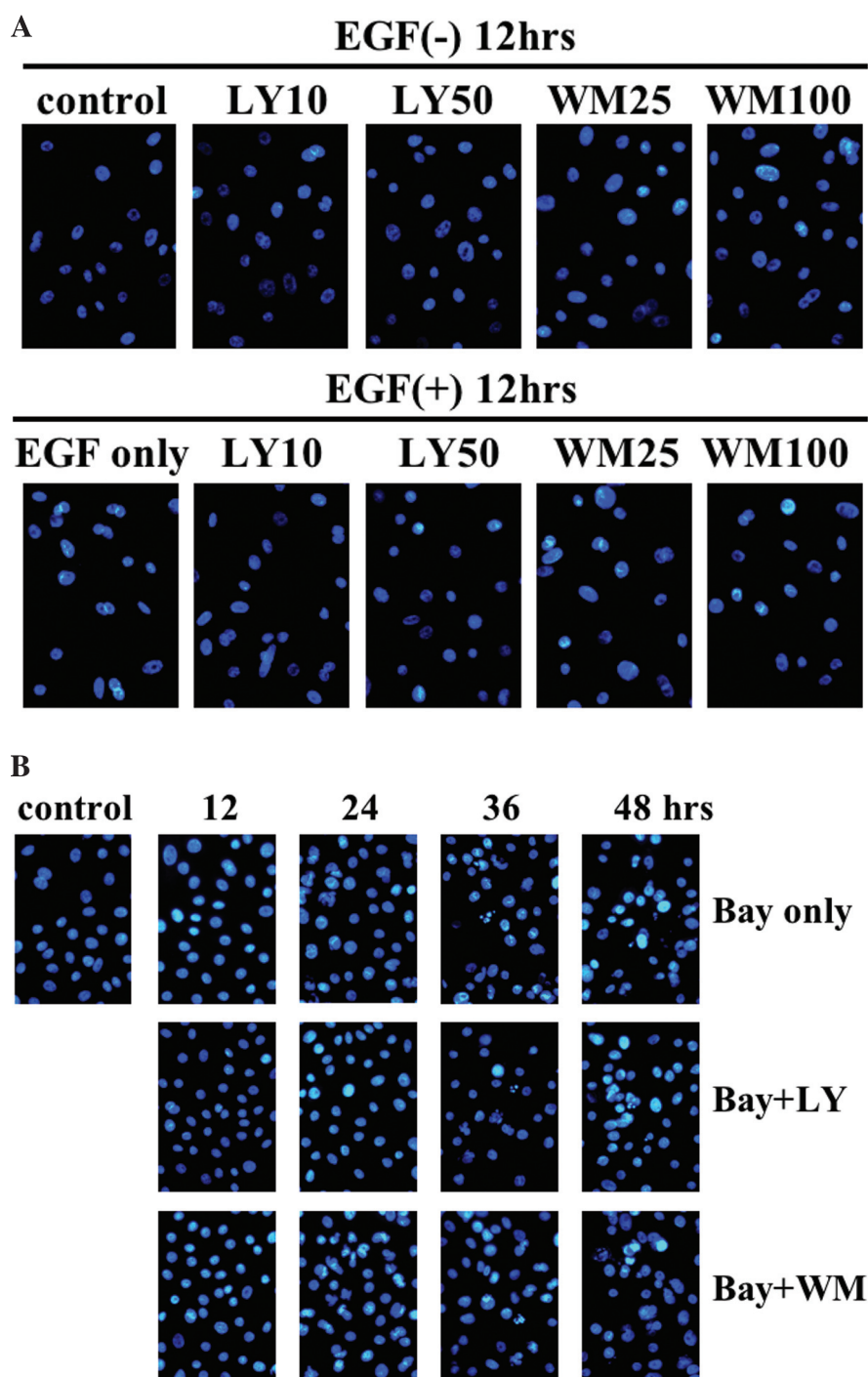


Fig. 1. Resistance to apoptosis of the primary cultured salivary gland epithelial cells (SGEC)

A. After 12 hours starvation of growth supplement, the primary cultured SGEC were treated with either 2 different concentrations of LY294002 (10 and 50 μ M) or wortmannin (25 and 100 μ M) in the presence or absence of EGF, for an additional 12 hours. Apoptosis in the nucleus was determined by Hoechst staining.

B. The primary cultured SGEC were then incubated with either 5mM of Bay 11-7082 only, Bay 11-7082 with 50 μ M of LY294002, or Bay 11-7082 with 100 μ M of wortmannin, for up to 48 hours, following the initial 12 hours starvation of growth supplement. Shown are representative results of two independent experiments (original magnification x200).

there was no detection of apoptosis (data not shown). Next, we stimulated the cells with either Bay 11-7082 or Bay 11-7082 with LY294002 or wortmannin

for up to 48 hours in order to evaluate whether or not inhibition of both the PI3K-Akt pathway and IKK-NF- κ B induces apoptosis in primary cultured

SGEC of SS patients (Fig. 1B). However, this attempt failed to induce any obvious cell death, presenting only one or two cell death during the stimulation.

Anti-Fas antibody needs stimulation of either PI3K inhibitor or IKK inhibitor to induce apoptosis to the primary cultured SGEC

In an effort to induce apoptosis in primary cultured SGEC, following growth factor starvation for up to 48 hours, we stimulated the cells with IgM anti-Fas antibody for 12 hours (Fig. 2A). However, the stimulation with anti-Fas antibody did not induce any apoptosis in cells. Therefore, we decided to stimulate the cells with anti-Fas antibody combined with either PI3K inhibitor or Bay 11-7082 (Fig. 2B). Although there was no significant apoptosis with the stimulation using LY294002, Bay 11-7082 or anti-Fas antibody alone, (Figs. 1 and 2A) combination of anti-Fas antibody and LY294002 for 12 hours clearly induced apoptosis in the primary cultured SGEC as demonstrated by chromatin condensation and fragmentation of nuclei observed by Hoechst staining (Fig. 2B lower panels). Apoptosis was also detected in the case of stimulation with anti-Fas antibody and inhibition of IKK with Bay 11-7082. To quantify the frequency of induced apoptosis, following Hoechst staining, we counted 100 of cells in each of the 3 different fields. Our results show that treatment with both anti-Fas antibody (CH-11) and LY294002 induced $25.0 \pm 2.6\%$ apoptosis in the primary cultured SGEC. This result was statistically larger than the frequency observed in the control cells (0.3 ± 0.6) ($p = 9.43 \times 10^{-5}$) (Fig. 2C). Furthermore, treatment with both anti-Fas antibody (CH-11) and Bay 11-7082 also induced apoptosis, which was significantly greater than that of the control cells ($6.0 \pm 1.0\%$). However, the p value ($p = 1.05 \times 10^{-3}$) in the latter was smaller than the case of treatment with both anti-Fas antibody (CH-11) and LY294002.

EGF has potential to inhibit apoptosis of the primary salivary epithelial cells
Our next focus of interest was whether or not apoptosis is inhibited by the

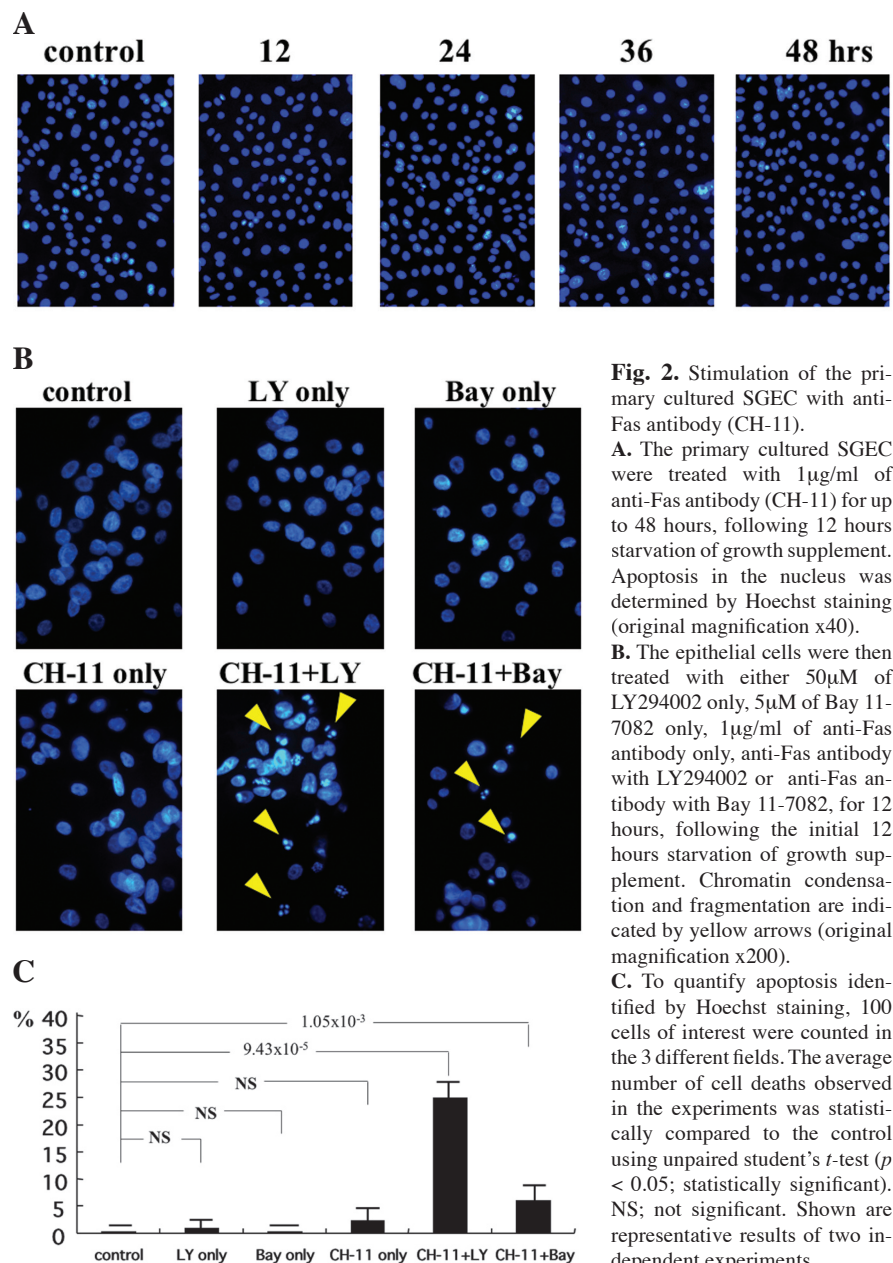


Fig. 2. Stimulation of the primary cultured SGEC with anti-Fas antibody (CH-11).

A. The primary cultured SGEC were treated with $1 \mu\text{g/ml}$ of anti-Fas antibody (CH-11) for up to 48 hours, following 12 hours starvation of growth supplement. Apoptosis in the nucleus was determined by Hoechst staining (original magnification $\times 40$).

B. The epithelial cells were then treated with either $50 \mu\text{M}$ of LY294002 only, $5 \mu\text{M}$ of Bay 11-7082 only, $1 \mu\text{g/ml}$ of anti-Fas antibody only, anti-Fas antibody with LY294002 or anti-Fas antibody with Bay 11-7082, for 12 hours, following the initial 12 hours starvation of growth supplement. Chromatin condensation and fragmentation are indicated by yellow arrows (original magnification $\times 200$).

C. To quantify apoptosis identified by Hoechst staining, 100 cells of interest were counted in the 3 different fields. The average number of cell deaths observed in the experiments was statistically compared to the control using unpaired student's t -test ($p < 0.05$; statistically significant). NS; not significant. Shown are representative results of two independent experiments.

addition of EGF. In the presence of both anti-Fas antibody and LY294002, we incubated the cells with EGF (Fig. 3A). Interestingly, the results demonstrated that dose-dependent addition of EGF inhibited apoptosis. In our previous study, 50 ng/ml of EGF effectively induced activation of Akt serine⁴⁷³ and NF- κB (16). In this present study, one fifth of that amount of EGF (10 ng/ml) was found to be sufficient to inhibit apoptosis. Finally, to quantify the inhibition of apoptosis, again, following Hoechst staining, we counted 100 cells in 3 different fields to detect apoptotic cells. Result show that the frequency of

apoptosis induced by both anti-Fas antibody and LY294002 (27.0 ± 4.4) was significantly reduced following the addition of 10 ng/ml EGF (0.3 ± 0.6) ($p = 4.6 \times 10^{-4}$). Also noted was a reduction with just 1 ng/ml EGF (13.6 ± 2.9) ($p = 0.011$) (Fig. 3B).

Inhibition of apoptosis by the effect of EGF indicated by TUNEL staining

Furthermore, to confirm whether or not apoptosis, induced by both anti-Fas antibody and Bay 11-7082, is inhibited by EGF, we employed TUNEL staining in order to observe double-stranded DNA breaks (Fig. 4). Both LY294002, and

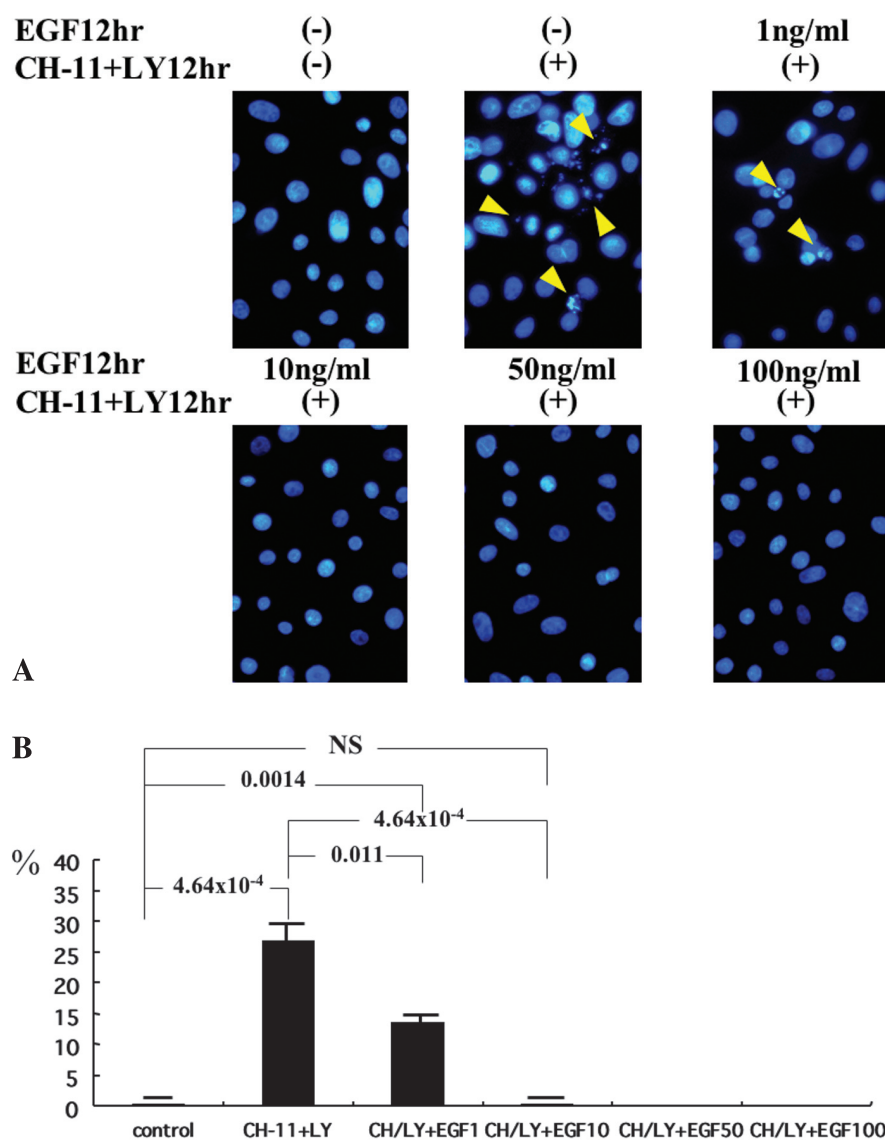


Fig. 3. Ability of EGF to inhibit apoptosis of the primary cultured SGEC.

A. The primary cultured SGEC were treated with both 1 μ g/ml of anti-Fas antibody and 50 μ M of LY294002 under the presence of different concentrations of EGF (1–100 ng/ml) for 12 hours, following growth supplement starvation. Apoptosis in the nucleus was determined by Hoechst staining. Chromatin condensation and fragmentation are indicated by yellow arrows (original magnification x200).

B. As before, to quantify apoptosis in identified by Hoechst staining, 100 cells of interest were counted in the 3 different fields. The average number of cell deaths observed as a result of stimulation of anti-Fas antibody with 50 μ M of LY294002 was statistically compared to the control using unpaired student's *t* test ($p < 0.05$; statistically significant). NS; not significant. Shown are representative results of two independent experiments.

Bay 11-7082, with anti-Fas antibody, induced TUNEL-positive staining indicated as yellow signals detected by confocal microscopy. Furthermore, the positive staining in both cases was completely inhibited by 50 ng/ml of EGF.

Discussion

Knowing the factors which modulate apoptosis in salivary epithelial cells of LSG of pSS patients is crucial for understanding the balance of cell death

occurs in the microenvironment. We previously reported the expression of both pro-apoptotic and anti-apoptotic molecules in LSG of pSS patients (10, 11, 18). The Fas/FasL system is a well-known pathway which induces apoptosis in various cells under the influence of cytokines (19). Previous reports (20, 21) have shown that apoptosis in human salivary glands can be induced through the addition of anti-Fas antibody and that the subsequent cell death

was enhanced by administration of cytokines such as tumor necrosis factor- α (TNF- α). However, Ping *et al.* recently reported that primary cultured SGEC of SS patients were not sensitive to single stimulation by anti-Fas antibody, CH-11 (22). Their study showed that apoptosis of primary cultured SGEC required other stimulation, including anti-CD40 antibody and gamma-interferon. These results implied that stimulation with anti-Fas antibody was not sufficient to induce apoptosis in primary cultured SGEC.

Although our approach of inducing apoptosis was different from that reported by Ping *et al.*, our results clearly show, in contrast to HSG cell lines, apoptosis of primary cultured SGEC appears to be mediated by other anti-apoptotic signaling pathways. PI3K-Akt is a known anti-apoptotic pathway in some cancer cell lines (23–25). Furthermore, it has been reported that in conjunction with this pathway, the presence of growth factors was necessary for the inhibition of apoptosis. Kakazu (26) *et al.* also reported that not the ERK, but the PI3K-Akt-1 pathway mediated inhibition of staurosporine-induced apoptosis of corneal epithelial cells by hepatocyte growth factor (HGF). Others have reported (27) that the anti-apoptotic effect of HGF was mediated by both PI3K-Akt and MAPK pathways. However, these reports also showed that the PI3K-Akt pathway was more influential than MAPK pathway with regard to mediating apoptosis. We previously reported (28, 29) that JNK and p38 were found in the infiltrating mononuclear cells in LSG of pSS patient. However, there was no obvious expression of these molecules in the salivary epithelial cells of pSS patients, suggesting that the PI3K-Akt pathway is more influential than the MAPK pathway in the mediation of apoptosis in salivary epithelial cells under the influence of EGF.

Regarding the anti-apoptotic effect of EGF through activation of Akt, Gibson *et al.* previously reported (30) that the PI3K inhibitor blocked both the EGF inhibition of Fas-mediated apoptosis, and Akt activation in breast adenocarcinoma cells. This suggested that Fas-induced high caspase activity was

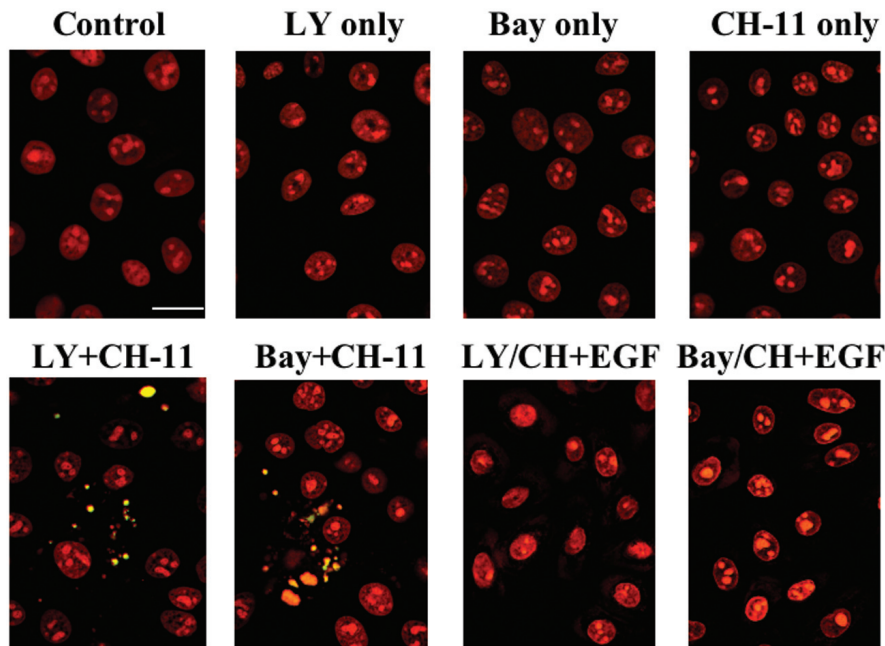


Fig. 4. Inhibition of apoptosis by the effect of EGF indicated by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining.

To confirm the chromatin condensation and fragmentation of the nuclei to be apoptosis, we employed TUNEL staining in order to observe double-stranded DNA breaks. The primary cultured SGEC were treated with either 50 μ M of LY294002 only, 5 μ M of Bay 11-7082 only, 1 μ g/ml of anti-Fas antibody only, or anti-Fas antibody with LY294002/Bay 11-7082 with or without 50 ng/ml of EGF for 12 hours, following growth supplement starvation for 12 hours. Positive-TUNEL staining with FITC-conjugated terminal deoxynucleotidyltransferase (TdT) was observed and recorded by confocal microscopy. Positive staining is expressed as yellow signals, in the merged view, following propidium iodide (PI) counter staining. (Bar 20 μ M).

suppressed by EGF. However, that activity was inhibited by wortmannin. We speculate that in the primary cultured SGEC there is a similar mechanism with an expression such as the inhibition of caspase activation.

As for the participation of NF- κ B as a downstream target of EGF stimulation, Biswas *et al.* (31) also demonstrated that EGF-EGF receptor interaction activated NF- κ B in estrogen receptor (ER) negative breast cancer cells. However, they showed the reduction of DNA binding activity of NF- κ B in the presence of LY294002, suggesting that NF- κ B is a downstream target of PI3K-Akt pathways. In the present study, inhibition of both PI3K-Akt pathway and NF- κ B had the potential to inhibit apoptosis although the apoptosis of the epithelial cells was restored by addition of EGF. Therefore, we suggest that both pathways play a crucial role in suppressing apoptosis in salivary epithelial cells of SS patients because we recently investigated that EGF clearly activates the PI3K-Akt pathway and NF- κ B (16).

In summary, our study suggests the potential of EGF to inhibit apoptosis through the activation of either the PI3K-Akt pathway or NF- κ B in primary cultured SGEC of pSS patients. However, other anti-apoptotic processes might be involved regarding Fas-mediated apoptosis of LSG because our observation demonstrated no more than 25% of apoptosis by removal of effect of Akt. For the practical clinical application of these theories in the treatment of sialadenitis of pSS patients, from the perspective of inhibition of apoptosis, further elucidation of the cell survival system will be necessary.

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