In Vitro Evidence of Nerve Growth Factor Effects on Human Conjunctival Epithelial Cell Differentiation and Mucin Gene Expression

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Purpose. Mucins released into the tear film are crucial to maintaining a healthy ocular surface. Alterations in goblet cell numbers and mucin secretion are observed in chronic ocular surface inflammatory diseases. Nerve growth factor (NGF) plays a crucial role in healing and inflammation of the ocular surface. The aim of this study was to evaluate in vitro the effect of NGF on conjunctival goblet cell differentiation and mucin production and secretion.

Methods. Human conjunctival epithelial cells were exposed to increasing NGF concentrations (1 to 250 ng/mL) and analyzed to quantify cell growth (MTT/Ki67/BrdU), goblet cell differentiation (PAS/MUC5AC confocal staining), and mucin mRNA expression (real-time PCR). Secreted and cellular MUC5AC were also analyzed by sandwich-ELISA and FACS, respectively. To confirm the biological effects of NGF, the same evaluations were performed on primary cultures, and changes in markers of stemness (p63) and commitment (14-3-3 sigma) were also investigated.

Results. In cell cultures, NGF induced a dose-dependent increase of goblet cell numbers, MUC5AC production, storage, and release. Additionally, in primary cultures, NGF induced an increase of abortive colonies and 14-3-3 sigma protein, and a decrease of p63 mRNA and protein, suggesting a differentiating effect of NGF on human conjunctival epithelium.

Conclusions. These findings show that NGF might play a role in the complex mechanism leading to conjunctival epithelium differentiation and mucin secretion. In addition to the known roles of NGF in promoting ocular surface healing and sensitivity, its effects on conjunctival goblet cells support a rationale to investigate the therapeutic effectiveness of NGF in dry eye disease. (*Invest Ophthalmol Vis Sci.* 2009;50:4622–4630) DOI: 10.1167/iovs.08-2716

The tear film plays a crucial role in maintaining a healthy conjunctival and corneal epithelium. It lubricates and protects the ocular surface from pathogens through a class of large hydrophilic glycoproteins called mucins.[1] Mucins are important structural and functional components of the tear film and are produced by corneal and conjunctival epithelia and by goblet cells, intercalated within the stratified conjunctival epithelium.[2] Membrane associated mucins, such as MUC1 and MUC4, are expressed by apical stratified epithelium,[3,4] while goblet cells produce, store, and release secretory mucin MUC5AC, the most prevalent large gel-forming secretory mucin.[4,5] MUC5AC, other than playing a role in the maintenance of a wet ocular surface, acts as clean-up/debris removing multimeric system, stabilizes fluids, harbors defense molecules secreted by the lacrimal gland, and provides a physical and chemical barrier that protects the ocular surface from infectious pathogens, desiccation, chemical, mechanical, and thermal trauma.[2,6–9] The importance of mucins in the maintenance of a healthy ocular surface is supported by the evidence that alterations of the ocular surface, including corneal injury and chronic inflammation, affect mucin production and goblet cells density.[10] Patients with dry eye syndrome, ocular cicatricial pemphigoid, Stevens-Johnson syndrome, and vitamin deficiency show mucin alterations and goblet cells loss in late stages of the disease.[10,11] In dry eye patients with Sjögren's syndrome, a significant reduction in MUC5AC— but not MUC1 and MUC4—mRNA and protein expression has been described, suggesting a crucial role of this mucin in drying diseases of the ocular surface.[10] Several authors have investigated the mechanisms regulating the production and release of MUC5AC by goblet cells to develop potential therapeutic strategy for dry eye. Physiologically, the main mechanisms to induce goblet cell secretion is neural stimulation, as demonstrated in rats by corneal debridement wounds.[12] In addition to the neuropeptides released by the nerve termini near the goblet cells, cytokines such as TNF-α, IL4, and IL9, and growth factors such as EGF and TGF-α, are potent inducers of MUC5AC production and/or secretion, supporting the hypothesis of a direct relationship between ocular inflammation and mucins alteration.[13] Despite the increasing number of studies on the mechanisms regulating mucin secretion by goblet cells, there is surprisingly little knowledge on the mechanisms regulating goblet cell differentiation and life cycle within the stratified conjunctival epithelia. It has been demonstrated that conjunc-

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tival goblet cells and conjunctival epithelial cells derive from the same stem cell, and that bipotency is also maintained in late transient amplifying cells.[14] Clonal analysis of human conjunctival epithelium shows that goblet cells differentiate from epithelial cells after a programmed number of cells doubling; however, there is no information regarding the factor(s) controlling the differentiation pathways.[14] The possibility that autocrine or paracrine factor(s), such as cytokines or growth factors, might induce switching during a specific time frame of the epithelial cell should be taken into consideration. Since goblet cell numbers change during chronic inflammatory diseases and corneal injury, the factors principally involved in these processes represent strong candidates to be investigated.

From this point of view, nerve growth factor (NGF) might represent an interesting candidate to be evaluated for its actions on conjunctival epithelial cell proliferation, differentiation, and mucin production. NGF is a pleiotropic factor that is released by structural (corneal and conjunctival fibroblasts and epithelial cells) and local/infiltrating immune cells (macrophages, lymphocytes, neutrophils, mast cells, eosinophils) during corneal healing and chronic inflammation of the ocular surface.[15] Normal human ocular surface epithelial cells synthesize and secrete biologically active NGF and express both NGF receptors $p75^{NTR}$ and $trkA^{NGFR}$.[15,16] After corneal injury, NGF levels are increased in both tears and cornea in animals and humans[17,18]; NGF concentration is increased in tears of patients with dry eye[19]; and NGF serum levels are higher in patients affected by vernal keratoconjunctivitis (VKC).[20] NGF receptors are overexpressed in patients affected by VKC and ocular cicatricial pemphigoid.[16] These chronic inflammatory diseases are characterized by important changes of the ocular surface, including mucins and goblet cells alteration.[11,21,22] In addition, it has been demonstrated that rat goblet cells express NGF receptors and in vitro NGF treatment induces a dose-dependent increase of goblet cells secretion.[23] Lastly, NGF treatment induces an increase of conjunctival goblet cells density and an improvement of tear film stability in an animal model of dry eye.[24]

In this study, it was evaluated in vitro whether NGF was able to influence mucin production, release, and goblet cell differentiation in a human conjunctival epithelial cell line that was previously characterized. Results were confirmed on primary human conjunctival epithelial cells.

Materials and Methods

Primary Antibodies and Other Reagents

Specific antibodies were mouse anti-human MUC5AC (sc-33667), rabbit anti-human $trkA^{NGFR}$ (2 μg/mL) or anti-human $p75^{NTR}$ (3 μg/mL), all purchased from Santa Cruz Biotech (Santa Cruz, CA). Conjugated secondary antibodies were as follows: species-specific FC/PE– coupled IgG antibodies, Cy2/Cy3–coupled $F(ab)_2$ antibodies, or POD-conjugated specific antibodies, all from Jackson Laboratories (West Grove, PA). Sterile tissue culture plasticware and analytical grade reagents were from NUNC (Roskilde, Denmark), SERVA (Weidelberg, Germany), ICN (Costa Mesa, CA), Euroclone (Milan, Italy), and Invitrogen-Gibco (Paisley, UK), unless specified otherwise.

The study was granted by the Intramural committee, in accordance with the tenets of the Declaration of Helsinki for in vitro studies.

Cell Line Culture and Study Design

The conjunctival epithelial cell line (Cj-ECs) was a kind gift of Ilene Gipson (Schepens Eye Research Institute, Boston, MA), whose characterization (gene/protein expression profile) and cell culturing were assessed and described previously in detail.[25]

Cj-ECs were cultured in keratinocyte serum-free medium (K-SFM; Gibco-Invitrogen Corp., Rockville, MD) containing 25 μg/mL bovine pituitary extract (BPE) and 10 ng/mL EGF in six-well plates (5×10^4 cells/cm^2) at 37°C in a 5% CO_2, followed by growth in a 1:1 mixture of K-SFM and low calcium dF12 (DMEM-F12; Euroclone). At confluence, Cj-ECs were switched to stratification medium (dF12 plus 10% FCS, 10 ng/mL EGF), and cultured for 0, 6, and 72 hours.

In specific experiments, Cj-ECs were cultured on collagen type I pre-coated plastic supports in dF12 without serum and EGF but with increasing NGF concentrations for the same time points. NGF was isolated from mouse submandibular glands and prepared according to the method of Bocchini and Angeletti.[26] Briefly, the submaxillary glands of adult male mice were explanted under sterile conditions, and the tissues were homogenized, centrifuged, and dialyzed. This aqueous gland extract was then passed through subsequent cellulose columns, thereby separating NGF by adsorption. The first step was gel filtration (Sephadex G-100 column; Roche Diagnostics, Mannheim, Germany) at pH 7.5, in which most of the active NGF was eluted in the 80,000 to 90,000 molecular weight range (designated the G-100 pool). The G-100 pool was then dialyzed at pH 5.0 and fractionated by CM52 cellulose chromatography at pH 5.0. The samples obtained were analyzed by spectrophotometry at a wavelength of 280 nm to identify NGF-containing fractions. Specificity of fractions was determined by Western blot analysis. NGF purity (95%) was estimated by high-performance liquid chromatography (HPLC; A-progel TSK3000PW-dp 10 mm, 7.5 mm inner diameter 630 cm; TSK, North Bend, WA) column equipped with a guard column calibrated with 40 mg of purified and bioactive murine 2.5S NGF standard. The NGF obtained was then dialyzed and lyophilized under sterile conditions and stored at −20°C until usage. Biological activity of purified NGF was evaluated by in vitro stimulation of neurite outgrowth in rat pheochromocytoma PC12 cells over a period of 7 to 14 days.

Cj-ECs were subject to specific characterization for NGF, $trkA^{NGFR}$ and $p75^{NTR}$ expression (see FACS/confocal/qPCR/NGF ELISA). Since the amount of mucins changed according to cell passage, we ran baseline experiments at every time-point.

Proliferation Assays

Cell proliferation was investigated at each experiment by counting either cells after brief enzymatic digestion (trypan blue exclusion test), or cells stained with the rabbit anti-human ki67 antibody (1:1000, Santa Cruz; fluorescent ABC technique, Vector Laboratories, Burlingame, CA), a nuclear proliferating factor ki67 recognizing all the cell cycles except G0.

Confocal Microscopy

Cj-ECs were grown on collagen type I pre-coated 4-well chamber slides (Nunc) and exposed to NGF for 6 hours or 72 hours. After stimulation, the cells were washed in Hank's balanced sodium salt (HBSS), fixed in 2% para-formaldehyde (PFA) in 100 mM phosphate buffer (PB), rinsed in PBS (10 mM PB–137 mM NaCl), quenched (50 mM NH_4Cl PBS), and finally permeabilized in 0.5% Triton X-100 PBS (TX-PBS). Monolayers were then probed with a mix of rabbit anti-human $trkA^{NGFR}$ and goat anti-human $p75^{NTR}$ antibodies. Specific binding of the primary antibodies was detected using secondary Cy2 or Cy3 $F(ab)_2$ antibodies (1/150 and 1/300, respectively) diluted in PBS containing 2% bovine serum albumin (BSA) and 0.05% Tween20. Control immune-staining was performed by substituting primary antibodies with control irrelevant IgG. Slides were closed with an anti-fade medium (Vectashield, Vector) and observed at the confocal inverted microscope (E2000U; Nikon, Tokyo, Japan). Brightness/contrast levels were optimized with the C1 software (Nikon) and images were prepared using imaging software (Adobe Photoshop 7.0; Adobe Systems Inc., San Jose, CA).

Immunoassay for NGF

NGF protein was evaluated in the conditioned media using a two-site NGF specific ELISA, showing absence of cross reactivity with BDNF or NT-3/4/5 and a sensitivity of 0.5 pg/mL.[27] As standard, murine βNGF (0.15 to 1 ng/mL; Alomone Laboratories, Jerusalem, Israel) was used.

Optical density (OD) was measured at λ_{450} (corrected for λ_{550}) by a microplate ELISA reader (Sunrise; Tecan Systems, Inc., San Jose, CA). The biological activity of Cj-EC-derived NGF was checked independently by using a PC12 bioassay, with or without neutralizing goat anti-NGF antibodies (30 μg/mL; R&D Systems, Minneapolis, MN).[27] Protein normalization was achieved by A280 spectrophotometer analysis (Nanodrop ND1000; Celbio, Milan, Italy) and equal protein loading was verified with GAPDH (Abcam, Cambridge, UK).

Relative Real-Time RT-PCR

Total RNA was obtained from 1×10^6 cells using a reagent (OMNIzol; Euroclone, Milan, Italy) and treated with DNaseI (AB1709; Ambion Inc., Austin, TX). RNA quality was analyzed by $\lambda_{260}/\lambda_{280}$ spectrophotometer analysis (Nanodrop) and 1% agarose gel electrophoreses. cDNAs were synthesized from 3 μg of total RNAs showing 260/280 1.8 and RIN 6, according to μ-MLV standardized procedure (Finnzyme, Milan, Italy) in a PTC-100 programable thermocycler (MJ Research, Watertown, MA). Three μL cDNAs for target gene (1 μL for referring GAPDH gene) were amplified in a 20 μL final volume of SYBR green PCR mixture (Applied Biosystems, Foster City, CA), using a real-time thermocycler (Opticon2; MJ Research). PCR amplification profile was one cycle of 95°C/15 minute, followed by 47 cycles at 95°C/30 seconds (denaturation), 58°C/25 seconds (annealing) and 72°C/30 seconds (elongation). Fluorescence was monitored at 60 to 90°C, 0.01°C for 0.3 seconds. Transcripts were finally incubated at 72°C/5 minutes. Only normalized samples (run in duplicate) were amplified and C_t values from the appropriate melting curves were used for statistical analysis. The specific pairs primers, the accession number, and the length of transcripts were as follows: MUC5AC (AF015521), for: TCC ACC ATA TAC CGC CAC AGA/ rev: TGG ACC GAC AGT CAC TGT CAA C, 103 bps; MUC1 (J05582), for: TTC CCA GCC ACC ACT CTG ATA C/ rev: AGT GCT GTG ATT GGA GGA GGT G, 116 bps; MUC4 (AF218265), for: TCC GTG TCC TGC TGG ATA ACC/ rev: GTT GCG GCT CAG GAG GAC TC, 105 bps; GAPDH (BC013310), for: GAA GGG GTC ATT GAT GGC AAC/ rev: GGG AAG GTG AAG GTC GGA GTC, 100 bps.[28] Primer pairs were designed by Primer3 software (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and prepared by MWG Biotech (Ebersberg, Germany). The mRNA complete sequence of each gene was obtained from the GenBank software (www.ncbi.nlm.nih.gov/GenBank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). The specificity of the primers was assessed by sequencing the resulting PCR products (Eurofins MWG Operon). In addition, conventional RT-PCR experiments were performed to confirm that only a single band is obtained when amplifying conjunctival cDNA with the primers used in this study (data not shown). Proper negative (without template or with total RNA) as well as positive controls (cDNA from reverse transcription of conjunctival total RNA) were produced for each run.[28]

Immunoassay for MUC5AC Protein

Western Blotting. Cells (1×10^6 cells) were dispase II-trypsin harvested and total proteins were extracted by 30 minutes' incubation with cold lysis-buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 7 μg/mL Aprotinin, and 1 mM PMSF) and subject to electrophoresis according to a standard procedure.[29] Equal protein amounts (40 to 80 μg) were fractionated on a 4% stacking/7% SDS-PAGE resolving gel at 160 V/60 minutes (Miniprotean3 apparatus; Bio-Rad), under reducing conditions (β-mercaptoethanol [β-ME]). Electrophoresed proteins were transferred to Hybond membranes under semi-drying conditions (12 V/45 minutes, BioRad). A molecular weight marker (6 to 210 kDa; SERVA) was run in parallel. Transferred proteins were stained with Ponceau S staining and probed with the monoclonal MUC5AC antibody, 1/700-diluted in TW-PBS, labeled with secondary POD-conjugated anti-mouse antibodies (1/20,000) and developed by ECL technique (SuperSignal West Pico Trial; Pierce, Rockford, IL) in a high performance imager station (Kodak 550; Eastman Kodak Company, Scientific Imaging Systems, Rochester, NY). Bands were digitally captured using the 1D Kodak Image Analysis Software, subjected to densitometric analysis and processed (Photoshop 7.0; Adobe). Membranes were stripped at 56°C/45 minutes in 1 M tris-buffer containing 2% SDS and 1.25 mM β-ME, re-blocked and probed with GAPDH antibodies (0.2 μg/mL; Abcam), to verify equal protein loading.

Sandwich-ELISA. According to previously published procedures,[30,31] the supernatants (50 μL from 1×10^6 cells) were diluted 1:1 with bicarbonate-carbonate buffer (pH 9.6), distributed in 96-well immunosorbant assay plates (NUNC), and left at 42°C until drying was complete. Plates were then rehydrated and blocked in 2% BSA, before the addition of MUC5AC antibody, 1/700-diluted in 0.05% TW-PBS. After a quenching treatment (0.3% H_2O_2 in PBS for 15 minutes), POD-conjugated anti-mouse antibodies (1/20,000) were added and the specific bound was developed with a ready to use TMB solution (Pierce). After HCl addition, optic density (OD) was detected at 470 to 570 nm in a microplate ELISA reader (Tecan Systems).

Histologic Analysis

Cj-ECs were grown on collagen type I–precoated round glasses (Miernfeld, Germany) and placed on 24-well plates to be exposed to increasing NGF concentrations. After brief PFA fixation, the cells were PBS washed and subject to the periodic acid-Schiff Kit staining, according to the manufacturers' instructions (Bioptica, Milan, Italy). Mucin-filled cells (goblet cells) were stained in bright-fuchsia on a dark-blue background.

FACS Analysis

Cj-ECs were dispase II-trypsin harvested and washed in HBSS without Ca^{2+} and Mg^{2+}. Single-cell suspensions (10^6 cells/well) were 0.3% PFA post fixed, methanol treated at −20°C, pretreated with 0.8% BSA −0.1% saponin HBSS, and incubated with mouse anti-human MUC5AC antibody (2 μg/mL), rabbit anti-human $trkA^{NGFR}$ antibodies or anti-human $p75^{NTR}$ antibodies, followed by specie-specific FC/PE coupled IgG–antibodies (1:500). Control, single, and double fluorescent staining were run in parallel for each set of experiments. Flow cytometric analysis was carried out with a dual-laser system (FACSCalibur; Becton Dickinson, San Jose, CA) using Macintosh-based CellQuest software. Logarithmic and linear signals were acquired from 5000 cells/sample. Mean fluorescence intensity (MFI) of linear distribution was calculated, aspecific signal for each sample was taken off the specific one, and results were expressed as increments relative to the controls, calculated as follows: ΔMFI = (specific MFI − aspecific MFI)/aspecific MFI. Histogram or density plots were arranged using the Win MDI 2.6 software. An MFI ratio of 1 represents no significant expression while an MFI ratio 1 represents higher levels of expression.

Primary Culture of Human Conjunctival Epithelial Cells

Conjunctival epithelial cells were obtained from healthy volunteers at the time of cataract surgery, and cultivated on a lethally irradiated feeder layer of 3T3-J2 cells as previously described.[32–34] Briefly, biopsy was treated with trypsin (0.05% trypsin and 0.01% EDTA) at 37°C for approximately 80 minutes. Cells were collected every 20 minutes, to obtain an average of 17.3×10^3 cells/mm^2. Cells were plated (1.5×10^4/cm^2) on lethally irradiated 3T3-J2 cells (2.4×10^4/cm^2) and cultured in 5% CO_2 and humidified atmosphere in DME and Ham's F12 media (2:1 mixture) containing FCS (10%), insulin (5 mg/mL), adenine (0.18 mM), hydrocortisone (0.4 mg/mL), cholera toxin (0.1 nM), triiodothyronine (2 nM), glutamine (4 mM), and penicillin/streptomycin (50 IU/mL). Epidermal growth factor (10 ng/mL) was added at 10 ng/mL beginning at the first feeding, 3 days after plating. Cultures were then fed every other day. Subconfluent primary cultures were passaged at a density of 6×10^3 cells/cm^2 and cultured as above. Conjunctival cells were subject to specific characterization for NGF, $trkA^{NGFR}$, and $p75^{NTR}$ expression

(see confocal and relative real time PCR). NGF concentration was evaluated by a specific ELISA in culture medium of conjunctival epithelial cells at different times.

To investigate the effect of NGF on proliferative compartment, exogenous NGF at concentration of 100 ng/mL was added to the culture medium (modified at a low FCS concentration of 1%). Cells (300–2000) from the biopsy and from each cell passage of serially cultivated mass and clonal cultures were plated onto 3T3 feeder layers and cultivated as above. Colonies were fixed 12 days later, stained with rhodamine B, and scored under a dissecting microscope. Values are expressed as the ratio of the number colonies on the number of inoculated cells. All colonies were scored whether progressively growing or aborted (total colony forming efficiency and percentage of aborted colonies values).

NGF effect on mucins production was evaluated by real-time PCR. Percentage of MUC5AC positive cells (goblet cells) was evaluated by confocal microscopy. To evaluate if NGF promotes differentiation of conjunctival epithelial cells, real-time PCR for p63 and confocal microscopy to evaluate percentage of p63 strongly positive cells and 14-3-3 sigma positive cells were performed.

Data Analysis

All experiments were repeated three times, each experiment being done in duplicate. Data are represented in the graph as mean ± SD. ANOVA followed by Tukey/Kramer post-hoc (multiple comparison) were carried out to determine significant differences ($P \leq 0.05$) between the groups (Statview II; Abacus Concepts, Inc, Piscataway, NJ). Fold increase/decrease expressions for mucin genes were calculated by REST software (http://nar.oupjournals.org/cgi/content/full/30/9/e36).[35]

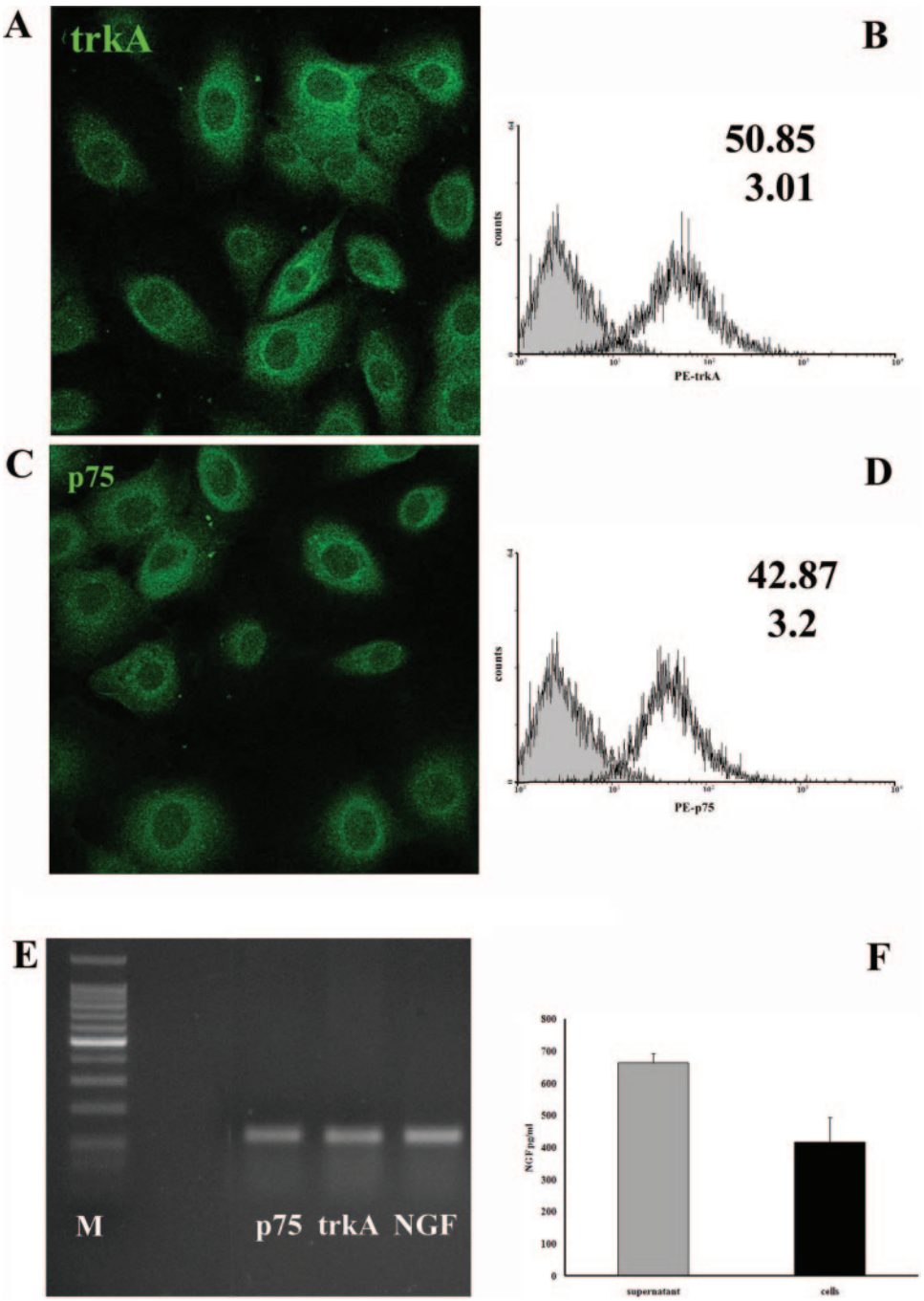


FIGURE 1. Cj-ECs express NGF, trkA^{NGFR}, and p75^{NTR} receptors. Cj-ECs express both trkA^{NGFR} and p75^{NTR} receptors when cultured for 24 hours in cKSFM/dF12 medium, and before the addition of NGF. (**A**, **C**) Immunofluorescence specific for trkA^{NGFR} and p75^{NTR}, respectively. Magnification ×600. (**B**, **D**) FACS analysis specific for trkA^{NGFR} and p75^{NTR}, respectively. *Shaded areas* show staining with isotype-matched control antibody; *bold lines* show specific antibody staining. Numbers in the right panels represent MFI values of trkA^{NGFR}/p75^{NTR} (*top*) and control (*bottom*) antibodies. (**E**) NGF, trkA^{NGFR}, and p75^{NTR} mRNAs expression by Cj-ECs. (**F**) NGF levels in Cj-ECs and supernatants as detected by ELISA. Figures show representative results from at least three experiments carried out in duplicate.

RESULTS

Characterization of Cj-ECs for NGF Pathway

Confocal analysis shows that Cj-ECs express both $trkA^{NGFR}$ and $p75^{NTR}$ (Figs. 1A, 1C). FACS analysis shows that the fluorescence intensity of each staining was comparable (Figs. 1B, 1D). In addition Cj-ECs produce, store (415.63 ± 78.47 pg/mL) and release NGF in their medium (663.33 ± 30.55 pg/mL), as demonstrated by PCR and ELISA (Figs. 1E, 1F).

NGF Modulation of Goblet Cell Mucin Production, Storage, Release, and Differentiation from Cj-EC

To examine whether NGF modulates MUC1, MUC4, and MUC5AC production, specific NGF incubations were carried out for 72-hours. Relative real-time PCR analysis revealed a dose dependent increase of both MUC5AC and MUC4 mRNA expression. In particular, a 7.01-fold increase expression of MUC5AC mRNA ($P < 0.05$; Fig. 2A) and a 3.50-fold increase of MUC4 mRNA were observed at the NGF concentration of 100 ng/mL ($P < 0.05$; Fig. 2B). On the contrary, NGF did not significantly affect MUC1 mRNA expression (Fig. 2B). This pattern of mRNA expression was similar as early as at 6-hour incubation (data not shown).

To evaluate whether NGF also modulates storage and release of MUC5AC, we investigated at the biochemical level its ability to induce MUC5AC by examining both secretion (sandwich-ELISA) and cellular (FACS/Western Blotting) content at 72 hours after NGF stimulation. NGF-treated Cj-ECs showed a significant release of MUC5AC in the conditioned media, compared with control cells (Fig. 3A). Cj-ECs treated with 100 ng/mL NGF expressed higher MUC5AC than the other untreated and treated cells, as showed by FACS (Fig. 3B) and Western Blotting (Fig. 3C). The higher MUC5AC cellular content was observed at 100 ng/mL NGF, in accordance with the protein content in the medium.

Since MUC5AC mRNA is specifically expressed/released by goblet cells, we investigated whether goblet cell differentiation occurred in our culture system. Therefore, Cj-ECs were grown at confluence according to the above reported protocol.[25] At confluence, the cells were shifted to a medium containing increasing NGF concentrations. Goblet cells were evaluated by morphologic and immunofluorescence analysis and quantified. After 72 hours, an increasing number of goblet cells (PAS and MUC5AC positive cells) was observed in cultures exposed to all the NGF concentrations tested (Fig. 4). The number of PAS and MUC5AC positive cells was significantly increased at 100 ng/mL and 250 ng/mL NGF, compared with medium containing 10% FCS (Fig. 4E). As showed in the inserts, PAS and MUC5AC staining were specifically localized within secretory granules (punctuate staining, high magnification; Fig. 4D).

The effect of NGF on mucins production and secretion was not associated with an increase in cell proliferation, as detected by MTT test, even though the cells showed a not significant increase in ki67 nuclear marker and intense PI staining in the nucleoli (data not shown).

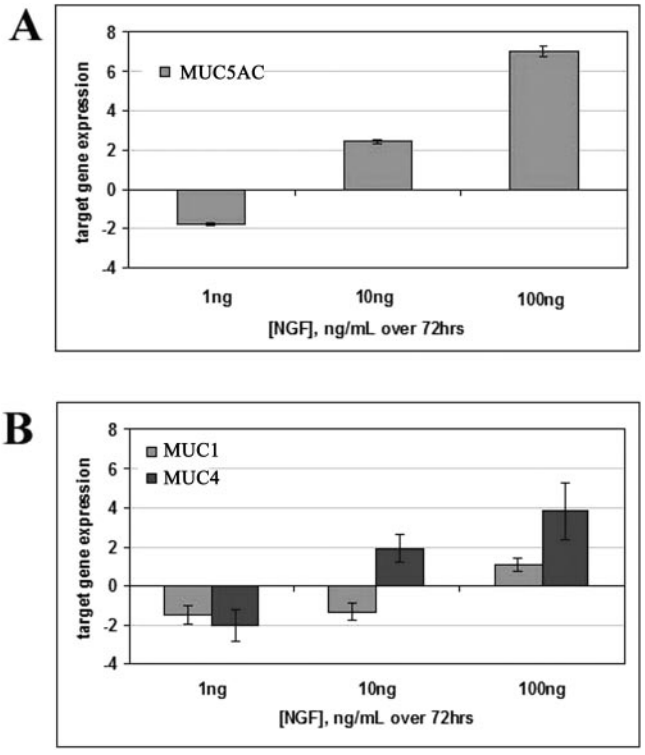


FIGURE 2. NGF induces specific mucin mRNA expression. Total RNA from Cj-ECs was reverse transcribed and amplified with mucin primers by real time PCR. (**A**) MUC5AC mRNA expression in cultures exposed to 100 ng/mL NGF over 72 hours; (**B**) MUC1 and MUC4 mRNA expression in cultures exposed to increasing NGF concentrations over 72 hours. Single melting curves for each transcript confirmed the specificity of the amplification (data not shown). Relative expression lower than two was considered not significant (P 0.05). Data were normalized to specific house-keeping mRNA (GAPDH) levels and expressed as fold change with respect to control cells (dF12/10%FCS). Analysis of single C_t was carried out and statistically evaluated by ANOVA-Tukey Kramer post-hoc analysis.

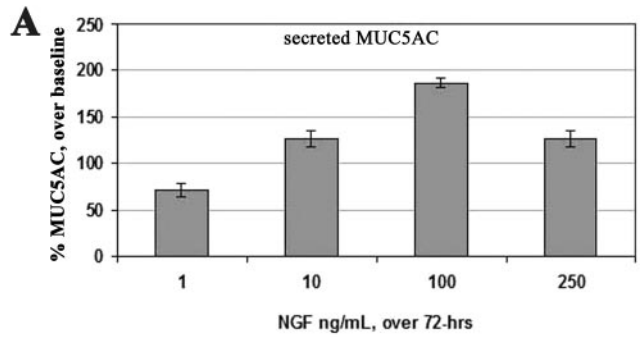
NGF Modulation of Goblet Cell Differentiation and Mucin Production in Human Primary Conjunctival Epithelial Cells

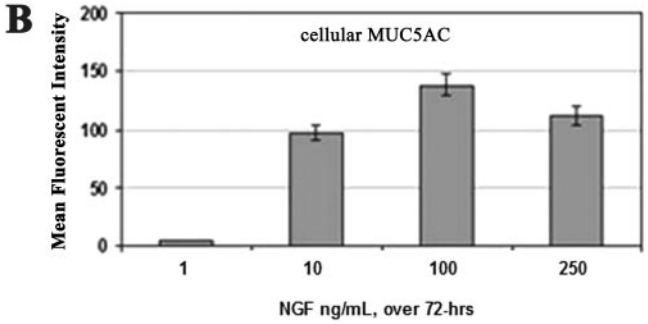
Primary cultures of epidermal and limbal cells generate cohesive epithelial sheets routinely used in cell therapy protocols. Similarly, human conjunctival cells cultured under identical conditions maintain virtually the same differentiation features and gene expression pattern of their in vivo counterpart.[32] We used such cultures to investigate NGF effects in a normal human experimental model. Cultured human conjunctival cells produced and released endogenous NGF in the culture medium in a time-dependent fashion and NGF synthesis was dependent on the keratinocyte growth phase (Fig. 5A). NGF secretion was maximal after the exponential phase of the growth and showed a three- to fivefold increase in the medium before confluence of both conjunctival cultures, with a corresponding increase in the total number of daughter colonies, scored as aborted colonies (Fig. 5B).

To examine whether NGF modulates differentiation of conjunctival epithelial cells, specific NGF incubations were carried out at 100 ng/mL in culture medium until confluence (about one week). Expression of markers as p63 for proliferative potential, 14-3-3 sigma for early epithelial differentiation, and MUC5AC for goblet cell differentiation, were compared in NGF-treated versus control cells.

NGF addition was associated with a decrease of p63 mRNA (compared with control cells) and percentage of p63+ positive cells (Fig. 5E), but was not associated with a decrease in cell yield, suggesting a role in regulating differentiation. According to this hypothesis, an increase of 14-3-3 sigma positive cells (Fig. 5F) associated with an increase of MUC5AC mRNA (Fig. 5C) and percentage of goblet cells (MUC5AC positive; Fig. 5D) was observed.

Finally, PAS staining confirmed at least a two-fold increase (compared with control cells) of goblet cells after NGF treatment, as shown also by MUC5AC staining.





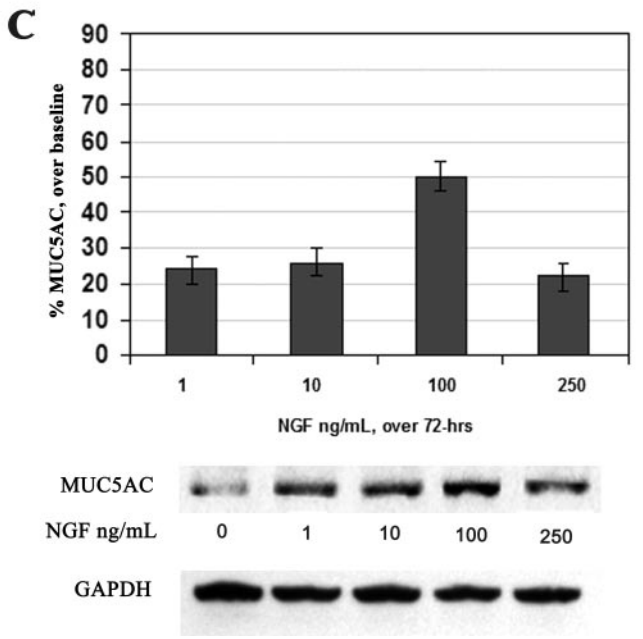


FIGURE 3. NGF increases both secretion and cellular content of MUC5AC. Biochemical analysis of MUC5AC in cultures grown in dF12+10%FCS in the presence of increasing NGF concentrations for 72 hours. (**A**) Representative results for MUC5AC protein secretion in the conditioned media, as detected by sandwich ELISA. Note the higher MUC5AC release at 100 ng/mL NGF. (**B**) The FACS analysis was carried out to quantify cellular MUC5AC (storage) in the same cultures. (**C**) Western Blotting shows an increased amount of cellular MUC5AC. Data are the mean ± SD of three experiments performed in quadruplicate. Error bars indicate SD.

On the contrary, NGF did not appear to induce any significant changes in MUC1 and MUC4 mRNA expression (Fig. 5C), suggesting a specific effect on goblet cell-specific MUC5AC. However, the results on MUC4 mRNA in primary cell cultures are in contrast with those obtained in Cj-ECs, where NGF stimulates MUC4 mRNA expression. This discrepancy may be related to different characteristic of the cells or by different cell culture conditions.

DISCUSSION

This study shows that NGF induces epithelial differentiation and increases in the number of goblet cells and MUC5AC mRNA/protein expression in both cell line and primary culture of human conjunctival epithelium.

The results of this study suggest that the effects of NGF on the ocular surface include a regulatory role in mucin regulation. In line with our findings, NGF treatment has been shown to induce an increase of tear production, stability, and goblet density in an animal model of dry eye.[24] In humans, refractive surgery induces changes of NGF levels that correlate with the degree of dry eye.[18] In addition, NGF treatment induces, other than corneal healing, an increase of tear production in 50% of patients affected by neurotrophic keratitis.[36,37] To date, these findings were explained by the well-known effect of NGF in stimulating sensory nerve leading to an improvement of ocular surface sensitivity and eliciting an increase of tear production.[15] Our results highlight a direct effect of NGF on the conjunctiva in promoting MUC5AC production and secretion. The effect of NGF in inducing mucin secretion by human goblet cells is consistent with a previous observation by Rios et al.,[23] who demonstrated that NGF, as well as BDNF, induced a

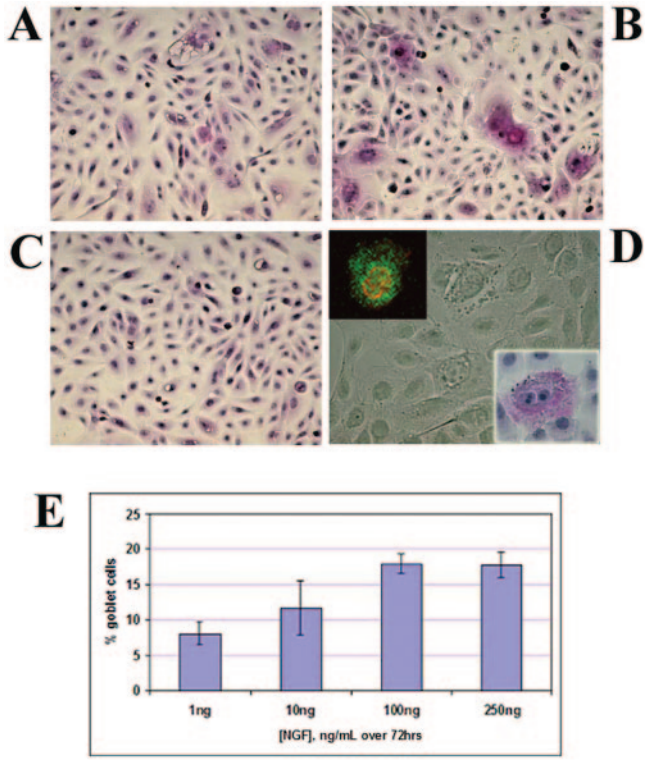


FIGURE 4. NGF modulates the differentiation of goblet cells. Light microscopy examination of cultures exposed to 10 ng/mL (**A**) and 100 ng/mL (**B**) NGF for 72 hours, compared with medium containing 10% FCS (**C**). The image in (**D**) shows a Normasky view (phase contrast acquisition) of the cultures and the *inserts* show a representative PAS positive cell (*bottom right*) and MUC5AC positive cell (*top left*). Goblet cells, as identified by PAS and MUC5AC positive staining, were increased by NGF exposure (**E**). Images are representative of three independent experiments carried out in triplicate. Magnifications: (**A–C**) ×200; (**D**) ×400; (*insert*) ×600.

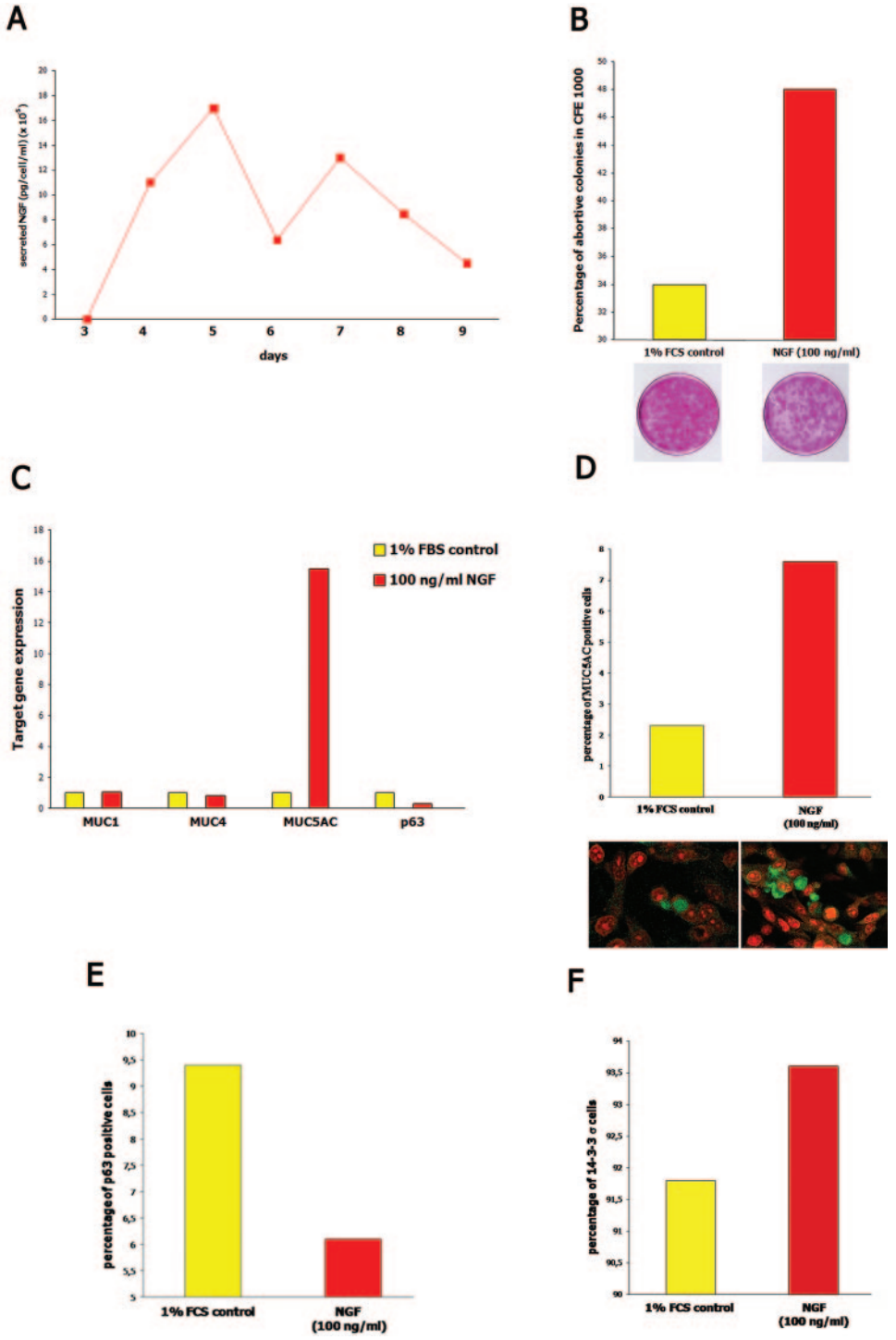


FIGURE 5. NGF effects on human primary conjunctival cell culture. The human conjunctival cells release NGF with the time point of maximum level on the fifth day in culture (**A**). NGF supplementation induces an increase of aborted colonies (**B**) and an increase of MUC5AC mRNA expression but not of MUC1 and MUC4 (**C**). The increase of MUC5AC mRNA was associated with an increase of the percentage of goblet cells (MUC5AC positive) (**D**). In addition, p63 mRNA (**C**) and p63 positive cells (**E**) were decreased. However, the percentage of 14-3-3 sigma positive cells was increased (**F**).

concentration-dependent stimulation of glycoconjugate secretion from rat cultured goblet cells in absence of cell proliferation.

Our study demonstrates that NGF induces not only production and secretion of MUC5AC, but also stimulates conjunctival epithelium differentiation. In fact, supplementation of conjunctival epithelial cells primary culture with NGF induced an increase of aborted colonies associated with an increase of 14-3-3 sigma expression and a decrease of p63 mRNA and protein. The two markers of commitment and proliferative potential of the ocular surface epithelial cells are 14-3-3 sigma and p63.[38,39] The evidence that NGF induces an increase of goblet cells in both primary culture and conjunctival cell line suggest that at least in part the differentiating effect of NGF on conjunctival epithelial cells leads to goblet cells differentiation. It has been demonstrated that: the conjunctival epithelial cells and the goblet cells are derived from a common bipotent progenitor[14,40]; commitment to differentiate into goblet cells occurs relatively late, so that goblet cells are generated by stem cell-derived transient amplifying cells[14]; and the decision of a conjunctival stem cell to differentiate into a goblet cell appears to be dependent on an intrinsic "cell doubling clock."[14] Usually, instructive or selective actions of external factors are evoked to explain the decision of a multipotent cell to enter a particular differentiation pathway. A selective action of envi-

ronmental factors implies that the initial choice of differentiated fate by a multipotent epithelial cell is controlled by a cell autonomous mechanism by secretion of growth factors that induce a specific set of sister cells to differentiate into goblet cells. NGF, produced and released by the epithelial cells, may be one of the factors responsible for the conjunctival cell fate decision through the binding to its receptors expressed by the same epithelial cells.[15] The differentiating action of NGF is well known in the nervous system.[41] Studies on neurosphere cells demonstrated the potent differentiation effects of NGF in inducing neuronal cells expressing ChAT+ and, to a lesser degree, tyrosine hydroxylase.[42] Indeed, NGF and its receptors are involved in progenitor cells maintenance/differentiation in bone marrow-derived cells and recently, it has been shown, in limbal cells.[43,44] These studies show that a differential ratio between $trkA^{NGFR}/p75^{NTR}$ expression characterizes the progenitor cells compared to the differentiated cells, suggesting that NGF as well as other $p75^{NTR}$-ligands may influence the cell to enter to a specific commitment.

From a clinical point of view, the effect of NGF in promoting mucin secretion and goblet cells differentiation offers an alternative explanation to several observations reported during ocular surface injury and inflammation and opens new therapeutic possibilities. In fact, in experimental animal models, corneal damage induces an increase of mucin production by the conjunctiva.[12] Based on the evidence that corneal injury induces an increase of NGF production and release,[17,18] it is reasonable to hypothesize that NGF, in concert with sensory neuropeptides, may modulate corneal wound healing and goblet cell secretion in response to physiopathological stimuli. For example, allergic diseases such as asthma and vernal keratoconjunctivitis are characterized by increasing circulating and local levels of NGF associated with an increase of mucin production.[20,45] On the other hand, in refractive surgery, decreased levels of NGF are associated with more severe signs and symptoms of dry eye.[18] Dry eye after refractive surgery is characterized not only by decrease of tear film, but also by reduction of mucin production and goblet cell density.[46] Apparently in contrast with these observations, it was previously published that patients with dry eye show increased tear levels of NGF.[19] However, the authors do not clarify the inflammatory state and integrity of the ocular surface, and it is known that corneal lesion and infiltrating immune cells are potent sources for NGF.[15] Consistent with this explanation, treatment with topical steroid induced a marked decrease of NGF levels associated with clinical improvement.[19] Other evidence, supporting a potential action of NGF in promoting mucin production in vivo, derives from the effect of NGF in promoting nerve survival and function.[41] The presence of NGF in the conjunctiva may represent an important factor for maintenance/activation of the autonomic nerves innervating the goblet cells.

In summary, NGF in the conjunctiva may exert pleiotropic effects: maintaining the survival of the epithelial cells including the goblet cells and the conjunctival nerves; promoting the neural innervation and maintaining the differentiation and survival of the "neural arc," made of afferent sensory and efferent parasympathetic and sympathetic nerves; and stimulating conjunctival goblet cell differentiation and mucin secretion.

These biological actions, associated with the effect of NGF in promoting corneal healing and in improving ocular surface sensitivity, make NGF a promising candidate for the treatment of dry eye.

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