Secretory Peptides TFF1 and TFF3 Synthesized in Human Conjunctival Goblet Cells

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PURPOSE. The objective of this study was to determine whether human conjunctival epithelium synthesizes TFF-peptides (formerly P-domain peptides, trefoil factors), a family of mucin-associated secretory peptides of the gastrointestinal tract.

METHODS. Expression of TFF-peptides in human conjunctiva was monitored by reverse transcription–polymerase chain reaction analysis. Antisera specific for TFF-peptides were used for immunohistochemistry to determine the presence and distribution of TFF-peptides in human conjunctiva.

RESULTS. mRNA expression of TFF1 and TFF3, but not TFF2, was detected in human conjunctiva. TFF1 and TFF3 but not TFF2 are stored in conjunctival goblet cells only as revealed by immunofluorescence.

CONCLUSIONS. Goblet cells of the human conjunctiva synthesize TFF1 and TFF3. These peptides, together with the secretory ocular mucin MUC5AC, may contribute to the rheological properties of the tear film. They also may influence healing of corneal wounds due to their motogenic properties. (Invest Ophthalmol Vis Sci. 1999;40:2220–2224)

The surface of the eye is overlaid by a complex tear film, which contributes optical clarity, lubrication, and a protective barrier against pathogenic and noxious agents. This film is approximately 35- to 45-µm thick and is composed of three layers: an outer lipid layer, secreted by the meibomian glands; an intermediate aqueous layer, secreted mainly by the lacrimal glands; and an inner mucus layer of approximately 30 µm containing mucins as its major structural component.¹,²

Mucins influence the rheological properties of the ocular mucus.¹,² The rheological properties are defined by the tear break-up time, which is changed in various pathologic conditions (e.g., in patients with dry eye symptoms). Alterations of mucin in human conjunctival epithelia of such patients have been reported.³

TFF-peptides⁴ (formerly P-domain peptides, trefoil factors) like mucins are typical constituents of mucus gels (e.g., from the gastrointestinal⁵–¹² and the respiratory ¹³ tracts), and amphibian integumentary mucins contain integral TFF-domains.¹⁴ Three TFF-peptides are known to exist in humans: TFF1 (formerly pS2), TFF2 (formerly hSP), and TFF3 (formerly hP1.B/hITF). They show distinct expression patterns; however, they all are typically secreted by mucin-producing cells (e.g., several types of goblet cells). TFF-peptides are thought to modulate the rheological properties of mucus gels by specific interaction with mucins.¹⁵,¹⁶ Furthermore, all three TFF-peptides are motogens that influence the migration rates of cell lines in wound healing assays in vitro¹⁷–¹⁹ and increase the resistance of animals against gastrointestinal damage in vivo.¹⁶,¹⁹–²⁴ This makes them candidates as factors regulating rapid repair of mucous epithelia by a process called restitution.⁸

TFF3 has recently been shown to be accumulated in goblet cells of porcine conjunctival epithelia, perhaps serving as a protective agent.²⁵ An analysis of TFF-peptides, expanded to the expression of all three members, in human conjunctival epithelial tissues is presented here.

MATERIALS AND METHODS

Antisera

The following antisera-monitoring TFF-peptides were used:

Anti-TFF1, a monoclonal mouse antiserum against the 30 C-terminal amino acids of human TFF1, was purchased from

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Zymed Laboratories (San Francisco, CA), and anti-TFF3, the polyclonal rabbit antiserum (anti-rTFF3-1) against the C terminus of the rat sequence, was described previously.\textsuperscript{15,26}

**RNA Extraction and Reverse Transcription–Polymerase Chain Reaction Analysis**

All investigations followed the tenets of the Declaration of Helsinki. Pieces of human conjunctivae were excised at the time of cataract surgery (2 men, 4 women; 65 to 88 years of age) for which informed consent was obtained from the subjects. All patients were free of preoperative chronic medication except one patient receiving topical eye medication for glaucoma. All conjunctiva obtained appeared normal at the time of preoperative examination. Freshly excised conjunctival bulbi tissue was immediately frozen in liquid nitrogen, and RNA was extracted from the pooled tissue samples using a guanidinium thiocyanate protocol. RNA purification via CsCl ultracentrifugation and reverse transcription–polymerase chain reaction (RT–PCR) analysis monitoring expression of TFF1, TFF2, and TFF3 were essentially as described previously,\textsuperscript{13} with 30 amplification cycles (Taq DNA polymerase; Boehringer Mannheim GmbH, Mannheim, Germany). As a control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts were amplified in a parallel reaction using a specific primer pair.\textsuperscript{13}

**General Histology and Immunofluorescence**

Human conjunctiva bulbi tissues were obtained in the course of cataract surgery (3 men, 1 woman; 70 to 83 years of age) or strabismus surgery (4 females; 6 to 31 years of age). None of the patients were using extended preoperative eye medication. Tissues were fixed in HEPES-buffered 4% paraformaldehyde overnight at 4°C, dehydrated in a series of graded ethanolos, and embedded in Technovit 7100 (Heraeus Kulzer GmbH, Wehrheim, Germany). Two-micrometer-thick sections were cut on a rotation microtome (Leica RM 2155), put on “Polysine” microslides (Menzel–Gläser, Braunschweig, Germany), and dried for 2 hours.

Mucins were stained using a combination of Alcian blue 8GX at pH 2.5 and the periodic acid–Schiff reaction as described previously.\textsuperscript{27} Nuclei were counterstained with hematoxylin.

For immunofluorescence, the fixed sections were treated with 0.1% papain (Merck, Darmstadt, Germany) for 15 minutes at room temperature, blocked with 1% bovine serum albumin for 20 minutes at 37°C and then incubated with the primary antibody (anti-TFF1, 1:5000 dilution, or anti-rTFF3-1, 1:1000 dilution) in 0.5% bovine serum albumin for 12 hours at 37°C. The secondary antibodies, Cy3-labeled sheep anti-mouse IgG (Dianova, Hamburg, Germany) or fluorescein-labeled sheep anti-rabbit IgG (Boehringer Mannheim), were incubated for 1 hour at 37°C. Furthermore, specificity of the staining was checked by competition with the corresponding peptides, that is, 1 ml anti-TFF1 (1:5000) or anti-rTFF3-1 (1:1000 dilution) was preadsorbed with 7 μg recombinant TFF1/dimer or 5 μg synthetic FKPLQEAECTF (representing the C-terminal of human TFF3), respectively, for at least 2 hours at 4°C and then used for immunofluorescence. DNA within the nuclei was stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma, Deisenhofen, Germany), and the sections were covered with fluorescent mounting medium (Dako, gmbH, Hamburg, Germany) after being washed in phosphate-buffered saline and water. For colocalization studies the sections were stained sequentially first with anti-rTFF3-1 (1:1000) and then with anti-TFF1 (1:1000). TFF3 staining was documented before TFF1 staining. Photomicrographs were taken on Kodak Ektachrome EPJ 520T.

**RESULTS**

**RT–PCR Analysis**

RNA was isolated from pooled conjunctival tissue of six patients of which cDNA was amplified by the use of specific primer pairs,\textsuperscript{13} testing for TFF1, TFF2, or TFF3 transcripts (Fig. 1A). TFF1- and TFF3-specific amplification products were clearly visible after separation on an agarose gel. In contrast, expression of TFF2 was not detectable in human conjunctiva. As controls, TFF1, TFF2, and TFF3 transcripts were monitored in parallel reactions with cDNA samples from human stomach and colon, respectively (Fig. 1B).

**Immunofluorescence Analysis**

The cellular localization of TFF1 and TFF3 revealed a similar pattern. Both peptides are stored exclusively in secretory granules of conjunctival goblet cells, which are Alcian blue-positive due to their characteristic mucin contents (Fig. 2). However, there are variations in the intensity between different goblet cells concerning their relative amounts of TFF1 and TFF3 (Fig. 3). Staining for TFF1 and TFF3 is shown to be specific because it could be competitively inhibited by the corresponding peptides in parallel sections (Figs. 2E, 2F). TFF2 could not be detected in all the conjunctiva samples tested.
FIGURE 2. TFF1 and TFF3 in human conjunctival epithelium. (A) Localization of TFF1 to goblet cells using the monoclonal TFF1 antiserum and immunoﬂuorescence with Cy3-label; counterstaining was with DAPI. (B) Localization of TFF3 to goblet cells using antiserum anti-rTFF3-1 and immunofluorescence with fluorescein-label; counterstaining was with DAPI. (C, D) Phase-contrast pictures of parallel sections to (A) and (B), respectively, stained with periodic acid–Schiff/Alcian blue. (E, F) No staining was observed in parallel reactions to (A) and (B) after competition with recombinant TFF1/dimer (E) or the synthetic peptide FKPLQEAECTF (F), respectively. Scale bars, 30 μm.

FIGURE 3. Sequential colocalization of TFF1 and TFF3 in human conjunctival goblet cells. (A) Localization of TFF1 using the monoclonal antiserum and immunofluorescence with Cy3-label after (C) having localized first TFF3 with anti-rTFF3-1 and fluorescein label. (B) Phase-contrast picture of (A, C). Scale bars, 30 μm.
using a specific polyclonal antiserum that successfully detected TFF2 in human stomach biopsies (data not shown).

**DISCUSSION**

Figures 1 through 3 clearly demonstrate biosynthesis and storage of the secretory peptides TFF1 and TFF3, but not TFF2, in the human conjunctival epithelium. This result is in agreement with a similar observation concerning TFF3 in porcine material. Both TFF1 and TFF3 are localized in the same conjunctival goblet cells, which does not conform to most other mucous epithelia that show a nonoverlapping distribution pattern of TFF-peptides (Table 1). For example, TFF1 is typically localized in gastric surface cells, whereas TFF3 is found in intestinal and respiratory goblet cells. Thus, the localization of TFF1 in conjunctival goblet cells is remarkable because one would have expected its expression in apical cells of the conjunctival epithelium (together with MUC4) by analogy with its gastric expression.

The localization of TFF1 and TFF3 in the human conjunctival goblet cells matches precisely that secreted by gastric surface cells, whereas TFF3 is found in intestinal epithelia that show a nonoverlapping distribution pattern of TFF-peptides and mucins. Thus, conjunctival TFF1 and TFF3 have to be considered typical mucin-associated peptides. Note that each mucin-producing cell type secretes a characteristic TFF-peptide/mucin combination, probably reflecting the complex physiological needs of the environment of these cells (Table 1).

In particular, the cocktail secreted by conjunctival goblet cells is a combination of that secreted by gastric surface cells and respiratory goblet cells.

It is postulated that TFF-peptides function as “link-peptides” interacting noncovalently with mucins and influencing the rheological properties of mucous gels. This hypothesis has been confirmed in preliminary studies with TFF2 and TFF3, both increasing the viscosities of mucin preparations. The dimeric structure of TFF1 and TFF3 is ideally suited to form an entangled network with MUC5AC in the ocular mucus. The precise nature of the interaction between TFF-peptides and mucins is currently not known; generally, protein–protein and protein–sugar interactions are imaginable. However, the latter has been proposed for TFF2 because of its established three-dimensional structure.

A second possible function of TFF1 and TFF3 in the eye is that of motogens by analogy to their wound healing properties in the gastrointestinal tract. After a wound in the cornea, migration of surrounding corneal epithelial cells is observed, whereas a total corneal epithelial defect can only be healed by conjunctival epithelial cells covering the denuded cornea. A corneal wound stimulates goblet cell mucus secretion in the same eye by activation of efferent parasympathetic and sympathetic nerves in the rat conjunctiva. This reflex secretion could also release TFF1 and TFF3.

Mucin-deficiency disorders such as ocular cicatricial pemphigoid, Steven-Johnson syndrome, or xerophthalmia could lower levels of TFF-peptides parallel to the decrease in goblet cell densities. In contrast, a higher number of goblet cells might enhance TFF-peptide secretion as when contact lenses are worn daily. TFF-peptide secretion might also be influenced by alterations in glycosylation of goblet cell mucins as they occur in patients with dry eye symptoms. However, as yet there are no data concerning synthesis and secretion of ocular TFF-peptides in pathologic conditions.

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**References**


