Trefoil Factor TFF1-Induced Protection of Conjunctival Cells from Apoptosis at Premitochondrial and Postmitochondrial Levels

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PURPOSE. Goblet cells of the conjunctival epithelium synthesize and secrete TFF1 (Trefoil factor 1), a small protease-resistant peptide that, together with mucins, is responsible for the rheologic properties of the tear film. This study aimed to determine whether TFF1, whose synthesis increases in inflammatory conditions such as pterygium, could protect conjunctival cells from apoptosis.

METHODS. Chang conjunctival cells, either wild-type or expressing TFF1 through stable transfection, were exposed to benznal-konium chloride (BAK) and ultraviolet (UV) irradiation to trigger apoptosis. The authors used cell fractionation to detect lipid raft–associated proteins, communoprecipitation to explore the formation of a death-inducing signaling complex (DISC), and a combination of immunofluorescence, immunoblotting, flow cytometry, siRNA-mediated decrease in gene expression, and electrophoretic mobility shift assay to explore the mechanisms of TFF1-protective effects.

RESULTS. TFF1 protects Chang conjunctival cells from apoptosis induced by UV irradiation and BAK at two levels. First, TFF1 prevents caspase-8 activation at the level of the DISC that involves Fas receptor in plasma membrane rafts, which in turn decreases the mitochondrial release of cytochrome c. Second, TFF1 interferes with caspase-9 and caspase-3 activation through an NF-κB–induced increase in the expression of XIAP (X-linked inhibitor of apoptosis protein).

CONCLUSIONS. TFF1 upregulation on inflammatory conditions may be a protective mechanism that limits conjunctival cell loss by inhibiting apoptosis upstream and downstream of the mitochondrial events. These observations suggest a potential interest of TFF1 or related peptides to prevent cell death in ocular surface disorders. (Invest Ophthalmol Vis Sci. 2008;49: 3790–3798) DOI:10.1167/iovs.07-1270

The cornea and the conjunctiva are stratified, squamous, nonkeratinizing epithelia covered by a stable tear film at the ocular surface. Ocular surface disorders, ranging from ocular irritation to defective wound healing and surface destruction, induce alterations in the tear film, whose defect is the hallmark of dry-eye disorders, and the epithelium, including conjunctival cell death. Epithelial cells die when they are severely damaged, such as by exposure to ultraviolet (UV) irradiation or to preservative-containing topical drugs. UV irradiation can induce photoconjunctivitis, photokeratitis, and pterygium, whereas long-term use of preservative can trigger conjunctival inflammation. We have previously described the differential pathways activated by UV irradiation and benznal-konium chloride (BAK), the most commonly used preservative in ophthalmic solutions, when triggering apoptosis of conjunctival cells.1

The epithelial surface of the eye and its specialized glandular infoldings produce the components of tear film, which include water, antimicrobial agents, cytokines, lipids, mucins, and trefoil factor (TFF) family proteins. Conjunctival goblet cells excrete gel-forming mucins, important components of the tear film, whereas conjunctival nongoblet cells express different types of transmembrane mucins on their superficial epithelial cells so that they can be wettable.2

Mucin-secreting epithelial cells also produce TFF proteins, which are small (6.5–12 kDa), protease-resistant proteins that have a clover leaf-like disulfide structure.2–4 Together with mucins, TFF peptides are responsible for the rheologic properties of the tear film.5 These peptides have demonstrated various other physiological functions, including packaging and secretion of mucins,6 promotion of epithelial migration, induction of cell scattering,7 activation of angiogenesis,8 and inhibition of apoptosis.9–12

Three tff genes clustered on chromosome 21q22.3 were identified in humans. The first gene, tff1, also known as ps2, was identified in a human breast cancer cell line.13 The other two human TFF family members are TFF2 (or spasmolytic polypeptide) and TFF3 (intestinal trefoil factor). The three TFF peptides are major secretory products of normal mucous epithelia, and each specialized mucosa secretes its specific cocktail of TFF peptides (i.e., the goblet cells of the conjunctiva secrete TFF1 and TFF3).14 We recently described an increased level of TFF1, but not TFF3, in pterygium, a benign inflammatory conjunctival tumor,15 which was in accordance with the potential role of TFF peptides in wounding responses and inflammatory processes.

In the present study, we explore the mechanisms of the protective effect of TFF1 toward proapoptotic agents in conjunctival cells. UV irradiation and BAK activate caspase-dependent pathways to chromatin nuclear condensation.1 We show that TFF1 negatively interferes with these pathways at two levels. The peptide delays caspase-8 activation at the level of the death-inducing signaling complex (DISC), upstream of the mitochondrial events. It also activates NF-κB and promotes the expression of the caspase inhibitor XIAP (X-linked inhibitor of apoptosis protein), which delays caspase-9 and caspase-3 activation by cytochrome c released from the mitochondria.

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**Experimental Procedures**

**Cell Culture, Transfection, and Treatment**

Chang cells (Wong-Kilburne clones 1-5c-4) obtained from the American Type Culture Collection (Rockville, MD) and cultured as described were seeded at 80% confluence for 24 hours prior to treatment with 4 μg/mL benzoalkonium chloride (BAK; Thea, Clermont-Ferrand, France) or irradiation with a 254-nm UV lamp at 30 J/m² (Merck, VWR International, Fontenay-sous-Bois, France). When indicated, cells were cotransfected with pUHD 10–3 plasmid encoding tff1 cDNA and pUHD172 to 1ne or were transfected with pBabe plasmid encoding a mutated IɛB (S52A and S60A; kindly provided by Salem Chouaib, INSERM U87, Villejuif, France) with the use of a transfection reagent (FuGENE 6; Roche Applied Science, Meylan, France), and antibiotic (Geneticin; Invitrogen, Carlsbad, CA)-resistant cell populations were selected.

**Antibodies and Chemical Reagents**

We used rabbit polyclonal antibodies (Abs) recognizing Bax (N20; Santa Cruz Biotechnology, Tebu-Bio, Le Perray en Yvelines, France); Bid and procaspase-9 (BD PharMingen, Heidelberg, Germany); caspase-3 and caspase-9 active fragments (Cell Signaling, Ozyme, Montigny le Bretonneux, France); PARP1 (Boehringer Mannheim, Indianapolis, IN); NF-κB p65, procaspase-3, Fas, and IɛB (Santa Cruz Biotechnology); mouse monoclonal Abs that recognize actin (Sigma-Aldrich, Saint Quentin Fallavier, France); caspase-8 (MBL, Cliniscience, Montrouge, France); caveolin 2 (clone 65; BD Transduction Laboratories, Lexington, KY); cytochrome c, RIP1, and FADD (BD PharMingen); Hsc70 (Santa Cruz Biotechnology); mitochondrial Hsp70 (Alexis, Lülfelfingen, Switzerland); TFF1 (P2802; kindly provided by MCR, INSERM, IGBMC, Strasbourg, France); and XIAP (SressGen, Tebu-Bio, Le Perray en Yvelines, France). We also used the IAP inhibitor embelin (Calbiochem, San Diego, CA) and the fluorogenic substrate (SressGen, Tebu-Bio, Le Perray en Yvelines, France); caspase-8 (MBL, Cliniscience, Montrouge, France); and dATP (Promega, Madison, WI) before analysis of caspases.

**Viability Assays**

Nuclear chromatin condensation, suggesting apoptosis, was identified by staining the nuclear chromatin of trypsinized cells with 10 μg/mL Hoechst 33342 (Sigma-Aldrich) before analyzing 100 cells in triplicate. Viability was also determined by incubating the cells for 5 minutes in ethanol before 15-minute labeling with methylene blue dye (100 mM boric acid, 25 mM disodium tetraborate, 120 mM NaCl, and 0.5 mg/mL methylene blue). HCl (0.1 M) was added before complete drying, and absorbance was read at 630 nm.

**Immunofluorescence Staining**

Cells on glass coverslips were fixed in 2% paraformaldehyde or in cold methanol before washing in cold acetone for NF-κB labeling. Cells were preincubated in Dulbecco phosphate-buffered saline (DPBS) with 5% bovine serum albumin (BSA) and then incubated with the primary antibody in DPBS with 2% BSA (with or without 0.1% saponin) for 90 minutes at room temperature. After washing, the cells were incubated for 40 minutes at room temperature with goat anti-mouse or anti-rabbit antibodies (Alexa 488, Molecular Probes, Eugene, OR) diluted in DPBS with 2% BSA. Nuclei were labeled with Hoechst 33342 before fluorescence microscopy analysis (Nikon, Champigny, France).

**Flow Cytometry Analyses**

Mitochondrial membrane potential was assessed by the retention of 3,3′-dihexyloxycarbocyanine (Dioc-6; Molecular Probes), a cationic and lipophilic fluorochrome that, at low concentrations, specifically accumulates in mitochondria, depending on the mitochondrial membrane potential. A decrease in green fluorescence reflects a decrease in mitochondrial membrane potential. Briefly, 10⁶ cells were incubated for 15 minutes at 37°C in the presence of 10 nM Dioc-6 solution before they were washed in PBS and analyzed on a flow cytometer (FACScan; Becton-Dickinson, Le Pont de Claix, France).

**Immunoblotting**

Cells were lysed for 15 minutes at 4°C in boiling buffer (10 mM Tris-HCl, pH 7.4, 1% SDS, 1 mM sodium vanadate, and 1:50 complete protease inhibitor). The viscosity of the sample was reduced by sonication. Mitochondrial and cytosolic fractions were prepared as described. Fifty micrograms of proteins were boiled for 5 minutes in loading buffer (125 mM Tris-HCl, pH 6.8, 10% β-mercaptoethanol, 4.6% SDS, 20% glycerol, and bromophenol blue) were separated on SDS-polyacrylamide gel and transferred onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Membranes were incubated overnight at 4°C with the primary antibody diluted in DPBS (DPBS with 0.1% Tween) and were washed three times before incubation with anti-mouse or anti-rabbit antibody coupled with horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA). Protein detection was performed with the use of an enhanced chemiluminescence detection kit (Santa Cruz Biotechnology).

**Immunoprecipitation**

Cells (100 × 10⁶) were incubated for 15 minutes at 4°C in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate) containing complete protease inhibitor mixture tablets (Roche Applied Science). After centrifugation for 10 minutes at 10,000g at 4°C, 1 μL goat antibody anti–caspase-8 (Santa Cruz Biotechnology) was added for 1 hour at 4°C before precipitating immune complexes using 30 μL mixed Sepharose 6B (Sigma-Aldrich) and G-Sepharose (Amersham Biosciences, Piscataway, NJ) for 16 hours at 4°C. The precipitate was washed four times with lysis buffer and boiled for 5 minutes in loading buffer before immunoblot analysis.

**Raft Isolation**

As described previously, 100 × 10⁶ cells were incubated for 30 minutes at 4°C in 1 mL MES buffer (25 mM MES, 150 mM NaCl, and complete protease inhibitor mixture) with 1% Triton X-100, before they were passed through an ice-cold cylinder cell homogenizer. lysates were placed on a linear sucrose gradient (5% and 40%) before centrifugation at 250,000g for 20 hours at 4°C, and 1-mL fractions (n = 12) were collected. Fractions 1 to 8 (450 μL) were precipitated by adding trichloroacetic acid (TCA, 5%) and were centrifuged for 10 minutes (8000g; 4°C), and the precipitate was resuspended in 60 μL loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5 mM EDTA, 100 mM dithiothreitol [DTT], 2 M urea, and 0.02% bromophenol blue). Thirty microliters of these fractions and 10 μL of the others were subjected to SDS-PAGE and immunoblotting. Results from caveolin-2–expressing fractions are shown.

**Cell-Free System**

Nuclei-free, mitochondria-free cytosolic extracts were generated as described. Briefly, 10⁶ cells were pelleted, resuspended in ice-cold buffer (20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 100 μM phenylmethylsulfonyl fluoride) and were allowed to swell for 20 minutes at 4°C. Then they were disrupted in a Potter Thomas homogenizer before centrifugation of lysates at 16,000g for 15 minutes at 4°C. Forty micrograms of proteins from the supernatant were incubated at 37°C with 5 μM horse heart cytochrome c (Sigma-Aldrich) and 1 mM dATP (Promega, Madison, WI) before analysis of caspases.

**Electrophoretic Mobility Shift Assay**

Electrophoretic mobility shift assay (EMSA) was performed as described. Briefly, nuclear fractions were collected and cleared by

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**Anti-apoptotic Effects of TFF1**

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with nuclear chromatin condensation. One representative image of each experiment is shown. Original magnification, ×40. (C) Cells transfected with the empty vector (Co) and those expressing TFF1 (T5 clone) were treated with 4 μg/mL BAK for indicated times or were exposed to UV lamp (30 J/m²) and analyzed at indicated times after irradiation. The percentage of cells with condensed chromatin (%). (D) Wild-type Chang cells were pretreated with 0.1 μM TFF1 recombinant peptide (T5 ▲, T9 ▻), then exposed to UV irradiation and observed at indicated times (hours) after irradiation. At each time, the percentage of cells with condensed nuclear chromatin was measured after cell labeling with Hoechst 33342. Each point is the mean ± SD of three independent experiments. *Results obtained in control cells (those transfected with an empty vector) are statistically distinct from results obtained in T5 or T9 clones. P < 0.001 (Student’s test). **Results obtained in nonpretreated cells are statistically distinct from those obtained in pretreated cells. P < 0.05 (Student’s t-test).
and TFF1-expressing cells underwent apoptosis when exposed to UV irradiation and BAK. However, the condensation of the nuclear chromatin, a hallmark of apoptotic cell death, was delayed in TFF1-expressing compared with control cells (Figs. 1B, 1C), which correlated with an increase in cell viability (Fig. 1C). Interestingly, a similar protective effect was observed when adding 0.1 μM recombinant human TFF1 to cell culture before UV irradiation (Fig. 1D).

We recently showed that UV irradiation and BAK induced redistribution of the type I transmembrane death receptor Fas in plasma membrane rafts and triggered the Fas-ligand-independent formation of a DISC that involved the adaptor molecule FADD (Fas-Associated Death Domain) and procaspase-8, thus leading to caspase-8 activation. Here, we confirm that both stimuli induce the redistribution of Fas, FADD, and procaspase-8 in caveolin-2-containing cell fractions, indicating membrane-associated lipid rafts (Fig. 2A). Coimmunoprecipitation experiments with an anti-caspase-8 antibody further showed that UV irradiation (Fig. 2B) and exposure to BAK (not shown) induced procaspase-8 interaction with FADD and Fas, suggesting recruitment of the enzyme into DISC (Fig. 2B). Stable expression of TFF1 in Chang cells did not significantly affect the redistribution of Fas, FADD, or caspase-8 into lipid rafts (Fig. 2A) but appeared to decrease slightly the quantity of FADD and Fas interacting with caspase-8 in response to UV irradiation (Fig. 2B).

On exposure to UV irradiation, the cleavage of two well-identified protein targets of caspase-8, namely the BH3-only protein Bim and the serine-threonine kinase RIP1, was delayed in Chang cells expressing TFF1 (Fig. 2C). The expression of TFF1 also decreased the ability of lysates from UV-irradiated Chang cells to cleave IETD-AMC peptide substrate (Fig. 2D). These two observations further suggested that TFF1 negatively interfered with caspase-8 activation. In addition, the cleavage of procaspase-8 into its 43-kDa active fragments induced by exposure to UV irradiation was delayed in TFF1-expressing compared with control cells (Fig. 2E). Together these results indicated that TFF1 did not prevent the redistribution of Fas into plasma membrane lipid rafts but negatively interfered with caspase-8 activation at the DISC level.

Expression of TFF1 also decreased the percentage of cells in which UV irradiation and BAK treatment induced the appearance of an active form of the proapoptotic, multi-BH domain protein Bax, as detected by using a conformation-specific antibody (Fig. 3A). It also decreased the percentage of cells in which these treatments induced the release of cytochrome c from the mitochondria to the cytosol, as determined by immunofluorescence (Fig. 3B) and immunoblotting (Fig. 3C), and...
the percentage of cells demonstrating a decrease in mitochondrial membrane potential, as identified by the use of the cationic and lipophilic dye, 3,3'-diethyloxocarbocyanine (Fig. 3D). Figure 3 demonstrates that TFF1 expression in conjunctival cells negatively interferes with the mitochondrial events that lead to apoptosis in response to UV irradiation and BAK treatment.

The release of cytochrome c and other soluble molecules from the mitochondria can activate a caspase cascade in the cytosol, starting with caspase-9 activation at the level of the apoptosome and leading to the activation of downstream caspases such as caspase-3 and to cellular protein cleavage.22,23 In accordance with the decrease in cytochrome c release, TFF1 expression in Chang cells prevented the appearance of DEVD- and LEHD-cleavage activities (Fig. 4A), the proteolytic cleavage of caspase-9 and -3 (Fig. 4B), and the cleavage of poly(ADP-ribose) polymerase 1, a well-known caspase substrate (Fig. 4C), in response to UV irradiation and BAK treatment. Similar results were obtained by adding recombinant human TFF1 to Chang cell culture before irradiation (not shown).

Based on our previous demonstration that caspase-8 activation was central in UV irradiation–induced conjunctival cell death,1 the ability of TFF1 to prevent caspase-8 activation may account for the negative impact of TFF1 on UV irradiation-
induced apoptosis. This explanation is insufficient regarding BAK-induced cell death because we showed that caspase-8 activation had little or no role in the death pathway activated by BAK in conjunctival cells.1 This led us to analyze whether TFF1 could interact with death pathways at another level, such as at the postmitochondrial level. We used a cell-free system in which cytochrome c and ATP were added to cytosolic extracts. In the presence of ATP, cytochrome c is known to trigger oligomerization of the adaptor protein Apaf-1, which recruits and activates caspase-9 in the so-called apoptosome. In turn, caspase-9 cleaves and activates downstream effector enzymes such as caspase-3.22,23 When cytosolic extracts were prepared from cells expressing TFF1, the ability of cytochrome c to induce the appearance of caspase-9– and caspase-3–active fragments was delayed (see Fig. 4D, 15 and 30 minutes).

IAPs are among regulators of the caspase cascades when activated downstream of the mitochondria. We observed that TFF1 expression through stable transfection increased the expression of XIAP protein (Fig. 4E) and mRNA (Fig. 4F) without affecting that of two other IAPs, c-IAP1 and survivin (Fig. 4F). Inhibition of XIAP by the IAP inhibitory molecule embelin (Fig. 5A) and the siRNA-mediated decrease in XIAP protein expression (Figs. 5B, 5C) sensitized Chang cells to UV irradiation–induced apoptosis. The siRNA-mediated decrease in XIAP expression also sensitized caspase-3 and caspase-9 to cytochrome c–mediated activation in the cell-free system obtained from
TFF1-expressing cells without affecting their response to cytochrome c in control cells (Figs. 5D, 5E).

Expression of the xiap gene was shown to be regulated at the transcriptional level by the transcription factor NF-κB,24 which signals the anti-apoptotic effect of TFF3 in intestinal cells.10 The canonic pathway to NF-κB activation involves the phosphorylation of I-κB, which targets its ubiquitination and degradation by the proteasome machinery, thus permitting NF-κB to translocate from the cytosol to the nucleus.25 The addition of TFF1 peptide (1 μM) to Chang cells induced the degradation of I-κBα, as demonstrated by immunoblotting (Fig. 6A), which was related to increased phosphorylation and proteosomal degradation (Fig. 6B), triggered the nuclear translocation of NF-κB (Fig. 6C), and stimulated NF-κB DNA binding, as demonstrated by EMSA (Fig. 6D). In Chang cells expressing a mutated I-κBα that prevents the activation of NF-κB, TFF1 did not induce XIAP overexpression (Fig. 6E).

**DISCUSSION**

Our results indicate that the increase in TFF1 expression identified in some conjunctival cells in an inflammatory context (e.g., pterygium) may protect these cells from death, such as...
through negatively interfering with caspase-mediated signaling pathways. TFF1 negatively interferes with caspase-8 activation in the DISC, which may account for the decrease in UV irradiation-induced cell death. TFF1 also directly interferes with postmitochondrial steps to death by inducing the overexpression of XIAP in an NF-κB-dependent manner, which may also account for the protective effects toward BAK-induced cell death.

A key question is whether this protective effect of TFF1 is limited to the fraction of conjunctival goblet cells that express the peptide or applies to neighboring cells through TFF1 exocytosis. Although we cannot definitively answer this question, we observed that recombinant TFF1 demonstrated a protective effect toward UV irradiation- and BAK-induced cell death and that the supernatant of TFF1-expressing Chang cells partially protected nontransfected cells from apoptosis induced by UV irradiation or BAK (not shown), suggesting that the secreted peptide could exert a protective effect on neighboring cells.

Extracellular TFFs are thought to act through hypothetical receptors postulated to localize on the basolateral membrane of mucous epithelia. 26 Their anti-apoptotic effect, demonstrated in various cellular systems and tissues, 9,11,12 was connected to their ability to promote cell migration because the cells must remain alive during this process. 27,28 During migration, cell survival can depend on the transcription factor NF-κB 10 and caspase inhibitors of the IAP family, 29 two effects depicted in conjunctival cells overexpressing TFF1. Activation of other signaling pathways probably contributes to the anti-apoptotic effects of TFF1, such as through activation of extracellular signal-related kinase (ERK) 1/2 and protein kinase B/Akt. 28,30 The protective and healing effects of TFF peptides, alone or in combination with epidermal growth factor (EGF), have been demonstrated in vivo in various types of intestinal damage, 51,52 and the luminal application was demonstrated to be superior over systemic delivery in this setting. 7 Our data suggest that therapeutic manipulation of TFF1 and related peptides could also be tested in ocular surface disorders to provide time for the cells to repair their damage rather than activating their death pathways.

References


