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 polypeptide 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 benzalkonium 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, immuno-blotting, 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 epithelial 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 benzalkonium chloride (BAK), the most commonly used preservative in ophthalmic solutions, when triggering apoptosis of conjunctival cells.

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 secrete 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.

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. Together with mucins, TFF peptides are responsible for the rheologic properties of the tear film. These peptides have demonstrated various other physiological functions, including packaging and secretion of mucins, promotion of epithelial migration, induction of cell scattering, activation of angiogenesis, and inhibition of apoptosis.

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. The other two human TFF family members are TFF2 (or spasmytic polypeptide) and TFF3 (intestinal trefoil factor). The three TFF peptides are major secretory products of normal mucous epithelia, and each specialized mucosa secretes its own specific cocktail of TFF peptides (i.e., the goblet cells of the conjunctiva secrete TFF1 and TFF3). We recently described an increased level of TFF1, but not TFF3, in pterygium, a benign inflammatory conjunctival tumor, 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. 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-Kilbourne 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 before treatment with 4 μg/ml benzalkonium chloride (BAK; Thea, Clermont-Ferrand, France) or irradiation with a 254-nm UV lamp at 30 J/m² (Merck, WKR International, Fontenay-sous-Bois, France). When indicated, cells were cotransfected with pUHD 10–3 plasmid encoding tff1 DNA and pUHD172 to Iec or were transfected with pBabe plasmid encoding a mutated Iec-B (S32A and S36A; kindly provided by Salem Chouaib, INSERM U487, Villejuif, France) with the use of a transfection reagent (FuGENE 6; Roche Applied Science, Meylan, France), and antibiotic (Genetixin; Invitrogen, Carlsbad, CA)-resistant cell populations were selected.

**Antigodies 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, Oxygen, Montigny le Bretonneux, France); PARP1 (Boehringer Mannheim, Indianapolis, IN); NF-κB p65, procaspase-3, Fas, and IκBα (Santa Cruz Biotechnology); mouse monoclonal Abs that recognize actin (Sigma-Aldrich, St. Quentin Fallavier, France); caspase-8 (MBL, Cliniscience, Montrouge, France); cavolin 2 (clone 65; BD Transduction Laboratories, Lexington, KY); cytochrome c, RIP1, and FADD (BD PharMingen), Hsc70 (Santa Cruz Biotechnology), mitochondrial Hsp70 (Alexis, Läufelfingen, 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 substrates Ac-DEVD-AMC, which mimics the preferred target site of caspase-3 and -7, Ac-LEHDA-AMC, which mimics the preferred target site of caspase-9, and zIETD-AMC, which mimics the preferred target site of caspase-8 (Biomol, Plymouth Meeting, PA). AMC (7-amino-4-methylcoumarin) and AFC (7-amino-4-trifluoromethylcoumarin) released from the substrate were excited at 380 and 400 nm to measure emission at 460 and 505 nm, respectively.

**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 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) before analyzing 100 cells in triplicate. Viability was also determined by incubating the cells for 5 minutes in lysis buffer (125 mM Tris-HCI, 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 TBBS (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-HCI, 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.

**Raff Isolation**

As described previously, 100 × 10⁶ cells were incubated for 30 minutes at 4°C in 1 ml MIES 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-HCI, pH 6.8, 2% SDS, 10% glycerol, 5 mM EDTA, 100 mM dithiothreitol [DTT], 2 M urea, and 0.02% bromophenol blue). Thirty micro liters 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 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).

**Immunofluorescence Staining**

Cells on glass coverslips were fixed in 2% paraformaldehyde or in cold methanol before washing in cold acetone. Nuclei were labeled with Hoechst 33342 before fluorescent microscopy analysis (Nikon, Champigny, France).

**Flow Cytometry Analyses**

Mitochondrial membrane potential was assessed by the retention of 3,3′-diethyloxacarbocyanine (Dioc-6; Molecular Probes), a cationic and anti-apoptotic Effects of TFF1
FIGURE 1. TFF1 expression in Chang cells prevents death induced by UV irradiation and exposure to BAK. (A) Chang cells were nontransfected (wild-type [WT]) or were stably transfected with an empty vector (Co) or the same vector encoding TFF1 (T5 clone) were treated with 4 μg/mL BAK for 16 hours or were exposed to UV lamp (30 J/m²) and analyzed at indicated times 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 the empty vector) are statistically distinct from results obtained in T5 or T9 clones. ∆Results obtained in pretreated cells. #Results obtained in nonpretreated cells are statistically distinct from those obtained in T5 or T9 clones.

RNA Interference

Two small interfering RNAs (siRNAs) specific for the xiap gene were used, starting at position 111 from the ATG codon (GUUUCAGC; Ambion, Austin, TX). As a control, we used scrambled siRNA (AGCAGCUGACCGGAUUAGU; Qiagen, Courtaboeuf, France). Exponentially growing cells were treated with a mixture of oligofectamine reagent (Invitrogen) and siRNA for 4 hours before adding culture medium.

RESULTS

We have shown previously that TFF1 was selectively and exclusively expressed in some goblet cells of the conjunctiva and that it accumulated in these cells in inflammatory situations. To determine the functional impact of TFF1 overexpression, we used the TFF1-negative Chang conjunctival cells, which were stably transfected with an empty vector or the vector encoding tff1 cDNA. One control and two TFF1-expressing cell populations were selected for further study (Fig. 1A). Control 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 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. #Results obtained in nonpretreated cells are statistically distinct from those obtained in pretreated cells. P < 0.001 (Student’s t-test). (D) Wild-type Chang cells were pretreated with 0.1 μM TFF1 recombinant peptide for 16 hours (∆) or were not pretreated (∗), 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 nonpretreated cells are statistically distinct from those obtained in pretreated cells. P < 0.05 (Student’s t-test).

with nuclear chromatin condensation. One representative image of each experiment is shown. Original magnification, ×40. (C) Cells transfected with the empty vector (Co) or the two clones of TFF1-expressing Chang cells were selected and named T5 and T9. Results shown are immunoblot (upper) and RT-PCR (lower) analyses of indicated proteins (TFF1, Hsc70) and genes (tff1, β2), respectively. Hsc70 (Heat shock cognate 70) was used as a loading control of the immunoblot. The β2 microglobulin (β2) gene was used as an RT-PCR control. (B) Cells transfected with the empty vector (Co) or those expressing TFF1 (T5 clone) were treated with 4 μg/mL BAK for 16 hours or were exposed to UV lamp (30 J/m²) and analyzed 8 hours later. Then the cells were labeled with Hoechst 33342 to identify those

centrifugation, and 5 μg proteins were incubated with γ²P-end (100,000 cpm; MP Biomedicals, Illkirch, France)–labeled NF-kB (AGTTGAGGGCCTTCCAGGC) consensus oligonucleotide (Promega) in a reaction buffer containing 10 μM HNB (0.5 M sucrose, 15 mM Tris, pH 7.5, 60 mM KCl, 0.25 mM EDTA, 0.125 mM EGTA, 0.15 mM spermin, 0.5 mM spermidine), 2 μM MgSp (10 mM MgCl₂, 80 mM spermidine), 1.5 μM NaPi (10 mM NaPi, 1 mM EDTA), 10 mM DTT, and 1 μL poly(dI-dC). After 30 minutes at room temperature, DNA-protein complexes were separated from free oligonucleotides by electrophoresis in a 4% polyacrylamide gel and detected by an imaging device (PhosphorImager; Amersham, Little Chalfont, Buckinghamshire, UK).

RT-PCR

RNA was prepared with the use of a kit (NucleoSpin RNA II; Macherey-Nagel, Hoerd, France), and cDNA was synthesized from total RNA by the use of Moloney murine leukemia virus reverse transcriptase (Invitrogen). PCR was performed with the use of Roche polymerase (AmpliTaq Gold; Roche Applied Science) and a thermal cycler (iCycler; Bio-Rad). Specific primers were synthesized for tff1 (forward, GAGAAGGCGGTGATCTG GCC; reverse, TGGTATXGATXGACAAGCC), β2 microglobulin (forward, ACCC CACCTGAAAAAGATGA; reverse, ATCTTCCAAACTCTCATGATG), and survivin (forward, CGAATGAAAGGCCAAGAGT; reverse, CAAGGCAGATTTAACCACAGG), xiap (forward, CTTGCAAGCTCAAAAGG; reverse, ATCTATCTAGCCGATGAC), and c-iap1 (forward, GUGGUAGUCCUGUUGC; Ambion, Austin, TX). As a control, we used scrambled siRNA (AGCAGCUGACGGGAUAGU; Qiagen, Courtaboeuf, France). Exponentially growing cells were treated with a mixture of oligofectamine reagent (Invitrogen) and siRNA for 4 hours before adding culture medium.

RESULTS

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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 Bid 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-AFC 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

![Figure 2](image)

**Figure 2.** TFF1 expression decreases caspase-8 activation at the DISC level. (A) Chang cells transfected with the empty vector (Co) or the tff1 gene expressing vector (T5 clone) were left untreated or collected 1 hour after UV irradiation or after a 3-hour exposure to 4 μg/mL BAK. Cell lysates were separated into 12 fractions on a sucrose gradient, and these fractions were analyzed by immunoblotting to detect the indicated proteins. Results shown are those obtained in fractions 3 and 4 (F3 and F4), which are the two main fractions that express caveolin-2, a protein specifically located in plasma membrane rafts. Results indicate that FAsz, FADD, and procaspase-8 (pro-C8) appear in plasma membrane rafts on exposure to UV or BAK and that overexpression of TFF1 does not affect this redistribution. (B) Chang cells transfected with the empty vector (Co) or the tff1 gene-expressing vector (T5 clone) were left untreated (untreated) or were collected 1 hour after UV irradiation (UV) for immunoblot analysis of indicated proteins, before (extracts) or after (IP) immunoprecipitation with an anticaspase-8 antibody. (C) Chang cells transfected with the empty vector (Co) or the tff1 gene-expressing vector (T5 clone) were left untreated (time 0) or 3 hours or 5 hours after UV irradiation for immunoblot analysis of indicated proteins. tBid indicated the truncated form of Bid. Actin, loading control. (D) Cells transfected with the empty vector (Co) and those expressing TFF1 (T5 and T9 clones) were collected before treatment (Untreated) or 6 hours after UV irradiation or after 16-hour exposure to BAK. IETD-AMC cleavage activity was measured in these samples. Results are the mean ± SD of three independent experiments. (E) Immunoblot analysis of pro-caspase-8 (54 kDa) and its intermediate cleavage fragment (43 kDa) in cells transfected with the empty vector (Co) and those expressing TFF1 (T5 clone) left untreated or collected at indicated times (hours) after UV exposure (UV) or treated with 4 μg/mL BAK for indicated times (BAK).
the percentage of cells demonstrating a decrease in mitochondrial membrane potential, as identified by the use of the cationic and lipophilic dye, 3,3'-diethyloxacarbocyanine (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.\textsuperscript{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,\textsuperscript{1} the ability of TFF1 to prevent caspase-8 activation may account for the negative impact of TFF1 on UV irradiation-

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**Figure 3.** TFF1 expression prevents apoptosis-associated mitochondrial changes. (A, B) Cells transfected as defined in Figures 1 and 2 (Co, empty vector; T5 and T9, tff1-expressing vector) were left untreated, were exposed to UV irradiation and examined 4 hours later, or were treated with BAK for 8 hours before immunofluorescence analysis of the active form of Bax (A) and cytochrome c (B). Upper: representative images; original magnification, ×60. Lower: percentages of cells with mitochondrial Bax (A) and percentages of cells with diffuse cytochrome c (B) were calculated. Results shown as histograms are the mean ± SD of three independent experiments. *Results obtained in T5 or T9 clones are statistically different from those obtained in control cells. P < 0.001 (Student’s t-test). (C) Immunoblot analysis of cytochrome c (cyc) in the mitochondria and the cytosol of control (Co) and TFF1-expressing (T5 clone) cells left untreated (UT) or collected 4 hours after UV irradiation (UV). Mitochondrial Hsp70 (mHsp70) was used as a loading control of the mitochondrial fractions. Molecular weights are indicated in kilodaltons. (D) Control (Co) and TFF1-expressing (T5 clone) cells were collected at indicated times after UV irradiation to measure by flow cytometry the accumulation of 3,3'-diethyloxacarbocyanine (Dioc-6) in mitochondria, which depends on the mitochondrial membrane potential. The decrease in fluorescence intensity, indicating a decrease in the mitochondrial membrane potential, is observed in control cells and is partially prevented by the overexpression of TFF1. Representative results of at least three independent experiments are shown.
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. 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. 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 cascade 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

**FIGURE 4.** TFF1 interferes with the postmitochondrial pathway to death by stimulating XIAP expression. (A) Chang cells, transfected as described in Figure 1, were left untreated (UT) or were collected 8 hours after UV irradiation (30 J/m²) or after 16 hours of exposure to 4 μg/mL BAK to measure the ability of cell lysates to cleave indicated fluorogenic substrates of caspases (LEHD and DEVD). Results shown are the mean ± SD of three independent experiments. (B) Immunoblot analysis of caspase-9 (C9) and caspase-3 (C3) in cells left untreated or collected at indicated times after UV irradiation or treated with BAK for indicated times. Molecular weights are indicated in kilodaltons. (C) Immunoblot analysis of poly(ADP-ribose) polymerase 1 (PARP1) protein in control (Co) and TFF1-expressing (T5) cells, left untreated (UT) or collected 4 hours after UV irradiation (30 J/m²) or after 12-hour exposure to 4 μg/mL BAK. Molecular weights are indicated in kilodaltons. The 85-kDa protein is a cleavage fragment of the 116-kDa native protein. (D) Immunoblot analysis of indicated proteins in cell-free extracts of control (Co) and TFF1-expressing (T5) cells incubated for indicated times with 5 μM cytochrome c and 1 mM dATP to activate caspase-9 and caspase-3. Hsc70, loading control. (E) Immunoblot analysis of XIAP in indicated cells. WT, nontransfected cells; Co, cells transfected with the empty vector; T5 and T9, tff-1-transfected clones; Hsc70, loading control. Molecular weights are indicated in kilodaltons. (F) Expression of indicated gene measured by RT-PCR in the same cell populations. β2 microglobulin (β2) gene was used as a control.
TFF1-expressing cells without affecting their response to cytochrome c in control cells (Figs. 5D, 5E).

Expression of the \textit{xiap} gene was shown to be regulated at the transcriptional level by the transcription factor NF-\textgamma/H9260B.\textsuperscript{24} which signals the anti-apoptotic effect of TFF3 in intestinal cells.\textsuperscript{10} The canonic pathway to NF-\textgamma/H9260B activation involves the phosphorylation of I-\textgamma/H9260B, which targets its ubiquitination and degradation by the proteasome machinery, thus permitting NF-\textgamma/H9260B to translocate from the cytosol to the nucleus.\textsuperscript{25} The addition of TFF1 peptide (1 \textmu M) to Chang cells induced the degradation of I-\textgamma/H9260B, as demonstrated by immunoblotting (Fig. 6A), which was related to increased phosphorylation and proteasomal degradation (Fig. 6B), triggered the nuclear translocation of NF-\textgamma/kB (Fig. 6C), and stimulated NF-\textgamma/kB DNA binding, as demonstrated by EMSA (Fig. 6D). In Chang cells expressing a mutated I-\textgamma/kB that prevents the activation of NF-\textgamma/kB, 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 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. Their anti-apoptotic effect, demonstrated in various cellular systems and tissues, was connected to their ability to promote cell migration because the cells must remain alive during this process. During migration, cell survival can depend on the transcription factor NF-κB and caspase inhibitors of the IAP family, 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. The protective and healing effects of TFF 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


