Thymosin-β4 Inhibits Corneal Epithelial Cell Apoptosis after Ethanol Exposure In Vitro

Gabriel Sosne,[1,2] Atif Siddiqi,[1] and Michelle Kurpakus-Wheater[1]

PURPOSE. The purpose of this study was to determine the effect of thymosin beta 4 (Tβ_4) treatment on human corneal epithelial cells exposed to ethanol in vitro. The efficacy of Tβ_4 in preventing mitochondrial disruption and in inhibiting caspase-mediated apoptosis was examined.

METHODS. Nontransformed human corneal epithelial cells (HCECs) at passage 4 were untreated or treated with ethanol (20% for 20 seconds) or a combination of ethanol and Tβ_4. The cells were allowed to recover from ethanol treatment for 24 hours. Mitochondrial membrane integrity and the release of cytochrome c to the cytoplasm were assessed using microscopy, Western blot, and ELISA. Bcl-2 expression and cell proliferation were measured using ELISA. Colorimetric activity assays were completed for caspase-2, -3, -8, and -9.

RESULTS. Tβ_4 treatment decreased deleterious mitochondrial alterations, significantly decreased cytochrome c release from mitochondria, and increased Bcl-2 expression in ethanol-exposed human corneal epithelial cells. In ethanol-exposed corneal epithelium Tβ_4 treatment inhibited caspase-2, -3, -8, and -9 activity, with caspase-8 showing the most significant inhibition. Tβ_4 treatment resulted in no significant effect on the proliferation of human corneal epithelial cells after ethanol exposure.

CONCLUSIONS. Tβ_4 plays an antiapoptotic role under conditions of epithelial cell challenge with an external stress such as exposure to ethanol. Tβ_4 may function as an antiapoptotic agent by inhibiting the release of cytochrome c from mitochondria and by suppressing the activation of caspases. (*Invest Ophthalmol Vis Sci.* 2004;45:1095–1100) DOI:10.1167/iovs.03-1002

The most common vision disorder in the United States is some type of refractive error. Accordingly approximately 120 million people require eyeglasses or contact lenses for vision correction.[1] Surgical options for correcting corneal refractive disorders include, but are not limited to, photorefractive keratectomy (PRK), laser in situ keratomileusis (LASIK), and laser-assisted subepithelial keratectomy (LASEK). The stated advantage of LASEK is reduction in postoperative pain, faster visual rehabilitation, and lessened probability of corneal haze.[2]

LASEK is based on the creation of an epithelial flap with 20% ethanol.[3] After laser ablation of the anterior stroma, the flap is repositioned over the corneal bed.[2] Alcohol is cytotoxic, and so a major concern with the use of ethanol in the cornea is continued viability of the epithelium after the procedure as it relates to wound healing and visual rehabilitation. This is especially relevant to LASEK because the epithelial flap covers the stromal surface after ablation. Measurements of the percentage of viable cells in an ethanol-treated corneal epithelial flap in human cadaveric eyes varied between 40% and 80%.[2,4] In immortalized human corneal epithelium, cell survival after ethanol treatment is highly dependent on exposure time and on the diluent used to prepare the 20% solution.[5] TUNEL-positive cells, indicative of apoptotic cell death, is evident at 8 and 24 hours after treatment in vitro with 20% ethanol for 20 or 40 seconds. This suggests that even a brief epithelial exposure to alcohol can activate apoptosis in the cornea.

Although evidence for ethanol-induced apoptosis in corneal epithelial cells has been reported, no further studies have been performed to identify mechanisms by which this may occur. Caspases are intermediates in cellular apoptotic cascades.[6] Caspases-2, -8, -9, and -10 are termed initiator caspases, and function upstream in activating the effector caspases. Caspase-8 is the major caspase that initiates death-receptor–mediated apoptotic cascades.[7,8] Caspase-9 is a major caspase for cytochrome c-mediated apoptotic mechanisms thought to be initiated by extracellularly applied stresses.[9,10] The effector, or executioner caspases include caspase-3, -6, and -7.[11,12] Ethanol has been reported to induce apoptosis through caspase-mediated pathways in T-cells,[13] neurons,[14] Leydig cells,[15] and liver hepatocytes.[16] Although it has been shown in several nonocular tissues that ethanol induces caspase-mediated apoptosis, the expression of caspases and their importance in corneal epithelial cell death processes after clinical application of ethanol have not been studied.

Thymosin β4 (Tβ_4) is a 4.9-kDa protein[17] originally thought to function primarily as a G-actin–sequestering protein.[17–20] However, in addition, topical application of Tβ_4 accelerates corneal wound healing after heptanol debridement in vivo, and stimulates corneal epithelial cell migration in vitro.[21] It stimulates the production of the extracellular matrix adhesion molecule laminin-5 in human conjunctival[22] and corneal[23] epithelial cells. Although the literature contains a few studies on how Tβ_4 may prevent apoptotic cell death,[24,25] nothing is known about its effects on corneal epithelial cell apoptosis.

We hypothesize that Tβ_4 treatment will significantly enhance epithelial cell survival in corneas receiving LASEK by inhibiting the initiation of apoptosis. In this study, we provided evidence to support this hypothesis by demonstrating that cells treated with ethanol and Tβ_4 compared with human corneal epithelial cells (HCECs) treated with ethanol alone exhibited decreased mitochondrial membrane alterations, decreased release of cytochrome c from mitochondria, and enhanced expression of Bcl-2. In addition, Tβ_4 suppresses the activity of caspases. Because survival of the epithelial cells contained in the flap is critical to uneventful postoperative healing and

successful vision correction after LASEK, our results suggest that Tβ_4 may be useful therapeutically after laser vision correction.

METHODS

HCEC Culture

Nontransformed HCECs frozen at passage 3 were purchased from Cascade Biologics (Portland, OR). The cells were rapidly thawed, plated, and maintained in serum-free culture medium containing human corneal growth supplement, as suggested by the manufacturer (EpiLife; Cascade Biologics). HCECs were used for experiments at passage 4. Tβ_4 was a gift from RegeneRx (Bethesda, MD) and was used at one concentration of 1000 ng/mL, because this concentration has been shown to be optimum for ocular surface epithelial cells in our previous studies.[21–23]

Morphologic Detection of Apoptosis

HCECs were plated in wells of a four-chamber microscope slide (Laboratory-Tek chamber slides; Sigma-Aldrich, St. Louis, MO). Cells in one chamber were treated with 200 μM hydrogen peroxide for 2 hours to induce apoptosis[26,27] and then replenished with serum-free medium. As a control, cells in the second chamber were not treated. Cells in the third chamber were treated with 20% ethanol in saline for 20 seconds, washed three times in culture medium, and replenished with fresh serum-free medium. Cells in the fourth chamber were treated with 20% ethanol in saline containing 1000 ng/mL Tβ_4 for 20 seconds, washed three times in culture medium, and replenished with medium containing 1000 ng/mL Tβ_4. Four, 8, and 24 hours after the treatments (only 24-hour results are shown), unfixed cells were incubated with 1 mL of a cationic fluorescent reagent (MitoCapture; Calbiochem-Novabiochem Corp., San Diego, CA) according to the protocol of the apoptosis detection kit. The cells were observed immediately using the rhodamine channel of a fluorescence microscope (Axiophot; Carl Zeiss Meditec, Dublin, CA). Images were then digitized and archived (Axiocam; Carl Zeiss Meditec).

Six images from each condition were used to quantify the number of viable cells. Viable cells were scored as having intact mitochondria visualized by the uptake of the reagent. For each treatment, the number of cells with viable mitochondria was divided by the total number of cells in the field, to calculate the percentage of viable cells. The average percentage of viable cells ± SEM was graphed. Statistical analysis was performed with the unpaired Student's t-test with significance set at $P \leq 0.05$.

Western Blot Analysis

HCECs at approximately 90% confluence were untreated or treated with hydrogen peroxide, ethanol, or ethanol and Tβ_4, as described in the previous section. HCECs were lysed with cytosol extraction buffer for 10 minutes on ice, using the protocol of the apoptosis assay kit (Cytochrome c Release Apoptosis Assay Kit; Oncogene Research Products, San Diego, CA). The lysates were centrifuged at 700g for 10 minutes, and the resultant supernatants were collected and centrifuged again at 10,000g for 30 minutes. These supernatants were collected as the cytosolic fraction. The pellet from the high-speed centrifugation was suspended in mitochondrial extraction buffer, according to the kit protocol and identified as the mitochondrial fraction.

The total protein concentration of each sample was measured using the bicinchoninic acid (BCA) method. Total protein (10 μg) was resolved by SDS-PAGE on 12% acrylamide gels. The proteins were electrophoretically transferred to nitrocellulose membranes and probed with a mouse monoclonal antibody provided with the kit that recognizes denatured human cytochrome c. Immunoreactive proteins were detected with alkaline-phosphatase–conjugated goat anti-mouse IgG coupled with a chemiluminescent protein detection system (Immun-Star; Bio-Rad Laboratories, Hercules, CA). Molecular weight mark-

ers obtained from Bio-Rad were run with the gels. The intensity of immunoreactive bands was determined by scanning the developed x-ray film and measuring the optical density as arbitrary units of integrated density value (IDV). Western blot analysis was repeated three times with similar results.

Cytochrome c ELISA

HCECs at approximately 90% confluence were untreated or treated with hydrogen peroxide, ethanol, or ethanol and Tβ_4, as described. Cells were lysed in preparation buffer (10 mM Tris-HCl (pH 7.5), 0.3 M sucrose, 10 μM aprotinin, 10 μM pepstatin A, 10 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride [PMSF]), according to the procedure given by the manufacturer of the ELISA kit (Cytochrome c ELISA kit; MBL International Corp., Watertown, MA). The lysates were centrifuged for 60 minutes at 10,000g, and supernatants were collected as the cytosolic fraction. Cytosolic fraction samples were then used for ELISA, to determine the concentration of cytochrome c in nanograms. To standardize for differences in concentration due to differences in cell number of the original cultures, total protein concentration in the cytosolic fractions was measured by using the method of Henkel and Bieger.[28] The ELISA was repeated in triplicate ($n = 3$ per replicate) to calculate the average nanograms cytochrome c in cytosolic fractions per milligram of protein ± SEM. Statistical analysis was performed using the upaired Student's t-test with significance set at $P \leq 0.05$.

Bcl-2 ELISA

HCECs at approximately 90% confluence were untreated or treated with ethanol, ethanol and Tβ_4, or 1000 ng/mL Tβ_4 only, as described. HCECs were lysed in resuspension buffer (50 mM Tris, 5 mM EDTA, 0.2 mM PMSF, 1 μg/mL pepstatin, and 0.5 μg/mL leupeptin) according to the protocol of a bcl-2 ELISA kit (Oncogene). Antigen extraction agent was added to the lysates, as suggested, and the cells were incubated on ice for 30 minutes. The cells were centrifuged at 10,000g for 10 minutes and defined as cytosolic fractions, and the cleared lysates were used for ELISA. The ELISA was repeated in triplicate ($n = 3$ per replicate) to calculate the average cytoplasmic bcl-2 in U/mL ± SEM. The results of the analysis were within the sensitivity range of the ELISA kit (5–200 U/mL). Statistical analysis was performed with the unpaired Student's t-test with significance value of $P \leq 0.05$.

Caspase Activity Assay

HCECs at approximately 90% confluence were treated with ethanol or ethanol and Tβ_4. Caspase-2, -3, -8, and -9 activities were measured with colorimetric activity assays with the appropriate p-nitroaniline conjugated substrate (Sigma-Aldrich). HCECs were lysed on ice for 10 minutes in buffer containing 50 mM HEPES (pH 7.4), 5 mM CHAPS (3-([3-cholamidopropyl]dimethylammonio-2-hydroxy-1-propanesulfonate), and 5 mM dithiothreitol (DTT). Samples of lysate (50 μL) were pipetted into wells of a 96-well plate. One hundred microliters of assay buffer (20 mM HEPES [pH 7.4], 0.1% CHAPS, 5 mM DTT, and 2 mM EDTA), 5 μL of the appropriate caspase substrate (10 mM final concentration), and 45 μL distilled water were also added to each well. Negative controls contained all reaction components except for cell lysate. After 2 hours' incubation at room temperature, detection was measured using a microplate reader at a 405-nm wavelength. Cell lysate was also used for total protein determination by the BCA method, and the microplate reader output corresponding to caspase activity was corrected for total protein. Caspase activity is reported as a percentage activity in HCECs treated with ethanol and Tβ_4 compared with HCECs treated with ethanol only (arbitrarily set at 100%). Triplicate assays were performed ($n = 6$ per assay) to calculate the average percentage activity ± SEM. Statistical analysis was performed using the unpaired Student's t-test with significance set at $P \leq 0.05$.

Cell Proliferation ELISA

HCECs were plated at 3000 cells/well in a 96-well microtiter plate and cultured in serum-free medium for 24 hours. Cells were untreated or

FIGURE 1. Immunofluorescence microscopy of corneal epithelial cells treated with a fluorescent dye reagent after treatment with hydrogen peroxide (**A**), 20% ethanol (**C**), or 20% ethanol and 1000 ng/mL Tβ_4 (**D**). Untreated control cells are shown in (**B**). Viable cells demonstrate punctuate labeling corresponding to reagent uptake by mitochondria (*arrows* in **B**, **C**, **D**). Apoptotic cells lack punctuate structures due to mitochondrial membrane depolarization (*arrowheads* in **A**, **C**, **D**). Bar, 100 μm. (**E**) Quantitative analysis of the effect of ethanol on corneal epithelial cell viability.
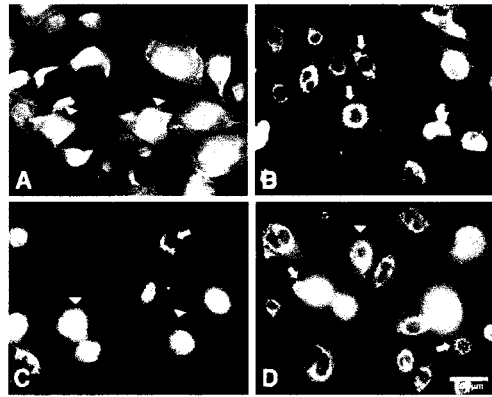
The (*) indicates a statistically significant decrease in cell viability in ethanol-treated HCECs compared with controls ($P < 0.0001$) and also compared with cells treated with ethanol and Tβ_4 ($P = 0.0010$).

treated with Tβ_4, ethanol, or ethanol and Tβ_4. After 20 hours of culture, 5-bromo-2′-deoxyuridine (BrdU) was added to each well to a final concentration of 10 μM. After 4 hours of incubation with BrdU (representing 24 hours of recovery time after ethanol treatment) the cells were fixed, and the DNA was denatured simultaneously with reagents supplied with a cell-proliferation ELISA kit (Roche Diagnostics, Indianapolis, IN). The cells were incubated with anti-BrdU peroxidase conjugate, washed, and incubated with color development substrate. The absorbance of the samples was measured at 450 nm wavelength (OD$_{450}$). The results of triplicate assays ($n = 8$ per replicate) are represented as mean optic density at 450 nm absorbance (OD$_{450}$) ± SEM. Statistical analysis was completed using the two-tailed unpaired Student's *t*-test with $P \leq 0.05$.

RESULTS

Effect of Tβ_4 on Ethanol-Treated HCECs: Morphologic Assessment of Apoptosis

In the studies described herein, nontransformed, early-passage HCECs were treated with 20% ethanol for 20 seconds, because this is a clinically and experimentally relevant protocol.[2–5,29–32] We initially investigated how ethanol exposure affects the membrane potential of mitochondria in corneal epithelial cells by using a cationic fluorescent reagent (MitoCapture; Calbiochem-Novabiochem Corp.). Reagent uptake by mitochondria in healthy cells is visualized as punctuate fluorescence throughout the cytoplasm. In contrast, apoptotic cells are incapable of accumulating the reagent in mitochondria due to membrane depolarization. Apoptotic cells are identified as lacking the punctuate structures, but instead have diffuse cytoplasmic fluorescence.

Cells were monitored 4, 8, and 24 hours after ethanol treatment. Evidence of apoptosis was not appreciable at 4 hours, while at 8 and 24 hours, our observations did not differ. Because of this, we present only results at 24 hours after treatment. HCECs treated with hydrogen peroxide, a reagent known to elicit apoptosis, showed evidence of extensive death, because no cells had the punctuate structures indicative of normal mitochondria (Fig. 1A). In contrast, untreated control HCEC cultures consisted of viable cells with the characteristic punctuate labeling (Fig. 1B). HCECs treated with ethanol demonstrated a mixed population of viable and apoptotic cells after exposure (Fig. 1C). HCECs treated with ethanol and Tβ_4 also showed a mixture of viable and apoptotic cells, although in general more healthy cells were observed (Fig. 1D).

Quantitative analysis, as shown in Figure 1E, demonstrated that compared with untreated control cells, HCEC treatment with ethanol resulted in a significant decrease in viable cells

(66% decrease, $P < 0.0001$). There was a similar significant decrease in viable cells in cultures treated with ethanol alone, compared with cells treated with ethanol and Tβ_4 (58% decrease, $P = 0.0010$). Although Tβ_4 could increase the number of viable cells in ethanol-treated cultures, it did not fully return levels to that of untreated control cells, because there remained a statistically significant decrease in viable cells treated with ethanol and Tβ_4 compared with the control cells (18% decrease, $P = 0.0167$).

Effect of Tβ_4 on Cytochrome *c* and Bcl-2 Expression in Ethanol-Treated HCECs

In addition to the disruption of mitochondrial membrane potential, apoptosis also triggered the release of cytochrome *c* from mitochondria to the cytoplasm. To provide additional evidence that Tβ_4 protects mitochondria from damage, we assayed cytochrome *c* release using immunoblot analysis and ELISA. The relative amount of cytochrome *c* in cytosolic and mitochondrial fractions of HCECs was determined semiquantitatively by Western blot analysis (Fig. 2). The ratios of cytosolic to mitochondrial IDV were used to calculate the percentage of cytochrome *c* that partitioned to the cytosolic fraction in each treatment. Correspondingly, 67% of cytochrome *c* was found in the cytosol in hydrogen-peroxide–treated HCECs, 34% in untreated controls, 75% in ethanol treated cells, and 45% in cells treated with ethanol and Tβ_4.

In a quantitative approach, cytochrome *c* concentration in cytosolic fractions was measured using a commercial ELISA (Fig. 3). Concentrations measured from the ELISA were corrected for total protein content of the samples to standardize for differences in cell number in the cultures. HCECs were treated with hydrogen peroxide to confirm that apoptosis correlates with an increase in cytosolic cytochrome *c* concentration. Compared with untreated control HCECs, cells exposed to ethanol demonstrated a significantly increased concentration of cytosolic cytochrome *c* (220% increase, $P = 0.0007$). In contrast, cytochrome *c* release from mitochondria
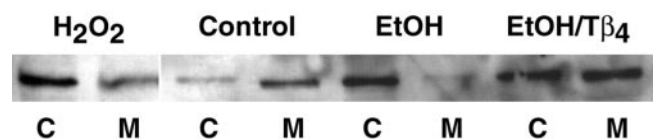

FIGURE 2. Immunoblot analysis of cytochrome *c* expression in cytosolic (**C**) and mitochondrial (**M**) fractions of corneal epithelial cells treated with peroxide (H$_2$O$_2$), ethanol (EtOH) or ethanol and Tβ_4 (EtOH/Tβ_4). Control cells (Control) are not treated.
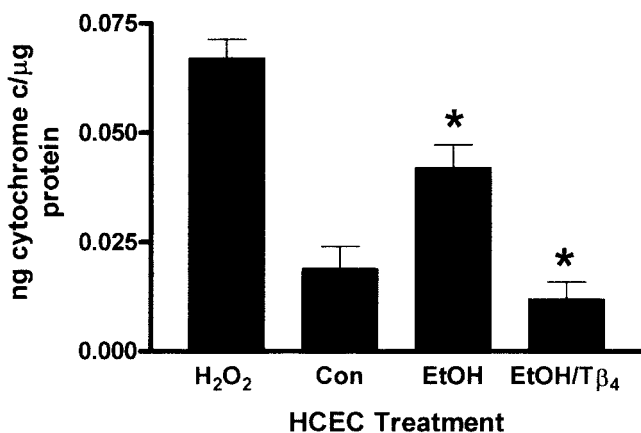
FIGURE 3. ELISA of cytochrome *c* content in the cytosolic fractions of HCECs treated with hydrogen peroxide (H_2O_2), ethanol (EtOH), or ethanol and $T\beta_4$ (EtOH/$T\beta_4$) Control cells (Con) were not treated. Cytochrome *c* concentrations were normalized to protein content in the samples. Peroxide-treated cells were analyzed to demonstrate apoptosis-induced release of cytochrome *c* into the cytosol. Compared with control, cells treated with ethanol demonstrated significantly increased cytosolic cytochrome *c* (*$P = 0.0007$). However cells treated with ethanol and $T\beta_4$ showed significantly decreased cytochrome *c* in the cytosol (*$P = 0.0022$ compared with control, $P < 0.0001$ compared with ethanol only).

to the cytoplasm was significantly decreased in HCECs treated with ethanol and $T\beta_4$ compared with cells treated with ethanol only (71% decrease, $P < 0.0001$) or compared with control cells (37% decrease, $P = 0.0022$).

The ability of $T\beta_4$ to abrogate ethanol-mediated apoptosis was further studied by investigating bcl-2 protein expression (Fig. 4). The major function of bcl-2 appears to be the inhibition of apoptosis, and expression of bcl-2 promotes cell survival.[33] In ethanol-treated HCECs, the concentration of cytosolic bcl-2 was not different from that of untreated control cells. In HCECs treated with ethanol and $T\beta_4$, however, a significantly increased concentration of cytosolic bcl-2 was observed compared with cells treated with ethanol only (138% increase,
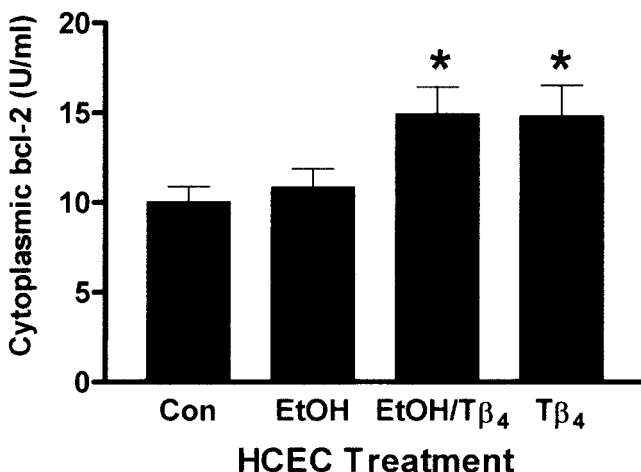

FIGURE 4. ELISA of bcl-2 protein content in the cytosolic fractions of HCECs treated with ethanol (EtOH), ethanol and $T\beta_4$ (EtOH/$T\beta_4$), or $T\beta_4$ only ($T\beta_4$). Control cells (Con) were not treated. Ethanol exposure did not affect bcl-2 expression in corneal epithelium. Cell treatment with $T\beta_4$, either in conjunction with ethanol or alone, resulted in a significant increase in cytosolic bcl-2 protein (probabilities are given in the Results section).
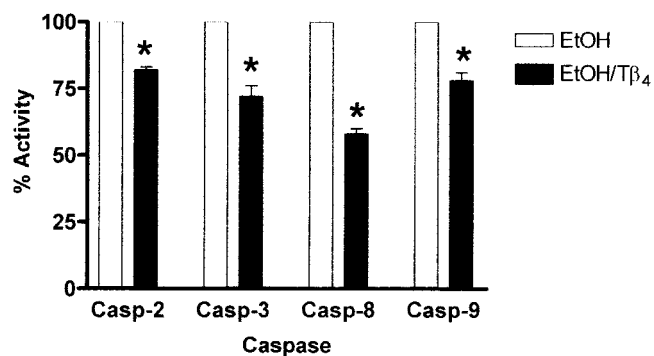

FIGURE 5. Caspase activity in corneal epithelial cells treated with ethanol or ethanol and $T\beta_4$. Activity in ethanol- and $T\beta_4$-treated corneal epithelium is expressed as average percent compared with ethanol-treated cells (arbitrarily set to 100%). For all caspases analyzed, the addition of $T\beta_4$ to the culture resulted in a significant decrease in activity (caspase-2, *$P = 0.0004$; caspase-3, *$P = 0.0026$; caspase-8, *$P < 0.0001$; caspase-9, *$P = 0.0012$). Note that inhibition of caspase-8 is greatest.

$P = 0.0482$) or compared with untreated control cells (149% increase, $P = 0.0490$). To provide additional support for the ability of $T\beta_4$ to stimulate bcl-2, HCECs were treated with $T\beta_4$ only and assayed for cytosolic bcl-2 protein. $T\beta_4$ treatment resulted in a significant increase in cytosolic bcl-2 compared with HCECs treated with ethanol only (137% increase, $P = 0.0442$) or compared with untreated control cells (148% increase, $P = 0.0397$).

Effect of $T\beta_4$ on Caspase Activity in Ethanol-Treated HCECs

Assays were completed to determine the effect of $T\beta_4$ on caspase activity in ethanol-exposed corneal epithelial cells in vitro. Colorimetric activity assays were used to determine the relative activities of caspase-2, -3, -8, and -9. The data are presented in Figure 5 as the percent caspase activity in HCECs treated with ethanol and $T\beta_4$ compared with cells treated with ethanol only. For all four caspases studied, cell treatment with $T\beta_4$ resulted in a significant inhibition of caspase activity (exact probabilities are given in the figure legend). However the greatest suppression as a consequence of $T\beta_4$ treatment was observed for caspase-8 activity.

Effect of $T\beta_4$ on Proliferation of Ethanol-Treated HCECs

To determine the effect of $T\beta_4$ on cell proliferation after exposure to ethanol, we performed a colorimetric BrdU-incorporation ELISA (Fig. 6). HCECs treated with ethanol demonstrated significantly inhibited proliferation after 24 hours of recovery time compared with untreated control cells (49% decrease, $P < 0.0001$). $T\beta_4$ did not positively affect mitotic activity, as proliferation did not differ between control and treated cells. In addition, HCECs treated with ethanol and $T\beta_4$ showed significantly inhibited proliferation compared with control cells (61% decrease, $P < 0.0001$). Cell proliferation in HCECs treated with a combination of ethanol and $T\beta_4$ was also significantly suppressed compared with ethanol-only treated HCECs (25% decrease, $P = 0.0004$).

DISCUSSION

After PRK and LASEK, it is important to preserve epithelial cell viability and integrity to achieve uneventful wound healing and optimal visual recovery. Dilute alcohol is frequently used for
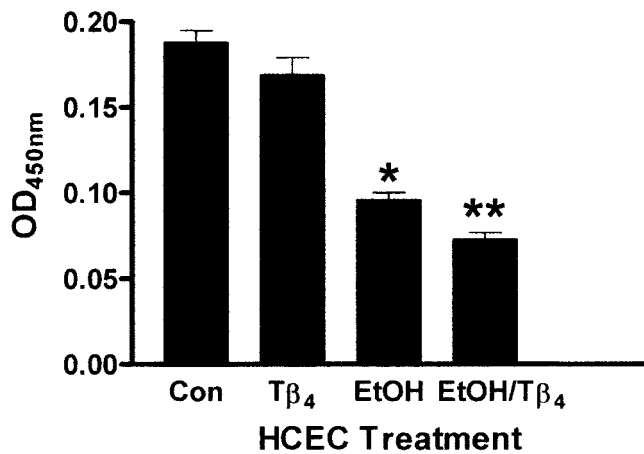
FIGURE 6. BrdU-incorporation cell proliferation ELISA of corneal epithelial cells treated with Tβ_4 (Tβ_4), ethanol (EtOH), or ethanol and Tβ_4 (EtOH/Tβ_4). Control cells (Con) were not treated. Cells exposed to ethanol were significantly less proliferative than control cells (*$P = 0.0001$). Similarly, cells treated with ethanol and Tβ_4 demonstrated significantly decreased proliferation compared with control cells (**$P < 0.0001$) or to ethanol-only treated cells (**$P = 0.0004$).

the removal of epithelium during PRK and LASEK.[3,29–32,34–37] Ethanol induces apoptosis in a variety of nonocular tissues,[38–43] and recent analyses have reported ethanol-induced apoptosis in corneal epithelium.[3,5] After wounding, especially with a noxious substance such as ethanol, the healing capacity of the epithelium should never be taken for granted. Epithelial defects, ingrowth, inflammation, and potential infection are all possible sequelae that may follow abnormal epithelial repair.

In the present study, we determined the ability of Tβ_4 to dampen the proapoptotic effect of ethanol on corneal epithelial cells in vitro. The results of our study demonstrate that after ethanol exposure, Tβ_4 decreases deleterious mitochondrial alterations, decreases cytochrome *c* release from mitochondria, increases bcl-2 expression, and decreases caspase activation in HCECs. Our results suggest that Tβ_4 has an antiapoptotic role, at least in circumstances where epithelial cells are challenged with an external stress such as ethanol exposure. Our observation agrees with the two known reports describing a relationship between Tβ_4 and apoptosis. Iguchi et al.[24] showed that apoptosis induced by anti-tumor drugs correlated with decreased Tβ_4 levels. In addition, it has been shown that cells that overexpress Tβ_4 demonstrate increased resistance to apoptosis.[25]

Caspase-9 is a major caspase for cytochrome *c*–mediated apoptotic mechanisms thought to be initiated by extracellularly applied stresses,[9,10] presumably including alcohol exposure. Our results support this notion by demonstrating altered mitochondrial function in corneal epithelium after ethanol treatment. Although Tβ_4 abrogates mitochondrial disruption and inhibits caspase activity in ethanol-treated corneal epithelium, the greatest inhibitory response was observed for caspase-8. This is surprising because caspase-8 is not generally considered to be an initiator caspase for stress-induced apoptosis.[7,8] Pastorino et al.[44] have shown that ethanol-exposed hepatocytes demonstrate mitochondrial depolarization and cytochrome *c* release to cytoplasm; however, they reported this to be a caspase-8 independent phenomenon. In contrast, the Fas/FasL and caspase-8 apoptotic pathway is suggested to play a primary role in acute ethanol-induced liver apoptosis in mice.[16,45,] In gastric mucosa, ethanol resulted in the activation of caspase-3, -8, and -9. Pretreatment of cells with an inhibitor of caspase-8 suppressed the onset of cell death.[46] The notion that ethanol induces apoptosis by different pathways depend-

ing on cell type is feasible, and further investigation is necessary to determine the precise roles of caspase-8, death ligands, mitochondrial changes, and caspase-9 in ethanol-induced apoptosis.

Nontransformed HCECs at passage 4 were used in the study described herein. Although our present report is limited to in vitro findings, the literature is replete with studies using cultured epithelial cells to reduce the number of animals used in preclinical evaluation of ocular toxicity of various substrates.[47–51] Because culture conditions cannot substitute for the complex nature of reparative processes that occur in the eye in vivo, we realize the potential limitations of our findings. Nevertheless, our results indicating increased cell survival with Tβ_4 treatment after ethanol exposure support growing evidence that Tβ_4 may regulate the wound-healing response to clinical advantage.[21,22,52,53]

Tβ_4 appears to suppress apoptosis in ethanol-exposed corneal epithelium but does not affect proliferation. Although it may seem surprising that suppressing apoptosis does not result in enhanced proliferation, one must realize that survival and proliferation are diverse cell processes. Although cell proliferation is one aspect of wound healing, cell migration and cell–extracellular matrix readhesion are vital as well. We have shown that Tβ_4 induces epithelial, but not keratocyte cell migration and increases the production of laminin-5, an extracellular matrix adhesion protein.[21–23] An agent that promotes corneal re-epithelialization, wound healing, and cell survival after trauma or surgery without adverse side effects would be a major clinical advance. As the mechanisms of action of Tβ_4 on wound healing are not fully understood, based on the presented findings herein, future studies will be aimed at discerning the mechanisms by which Tβ_4 inhibits caspase activation and subsequent cellular apoptosis. Studies are also planned to examine how Tβ_4 may effect corneal epithelial-stromal interactions through cytokine and chemokine expression during the repair process after wounding. Elucidation of these mechanisms may provide new insights into the documented reparative properties of Tβ_4.

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