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Protection of Thymosin Beta-4 on Corneal Endothelial Cells from UVB-Induced Apoptosis

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Abstract

Cornea absorbs most of daily ultraviolet (UV) light. An excess of UV damages results in not only keratopathy and cataract but also maculopathy. It has been reported that thymosin beta-4 ($T\beta_4$) promotes wound healing, decreases inflammatory response and prevents apoptosis of corneal epithelial cells. However, it is not clear whether $T\beta_4$ protects UVB-induced corneal injury, particularly in corneal endothelial cells because of its non-proliferation in nature. The purpose of this study is to compare the protective effects of $T\beta_4$ on bovine corneal endothelial (BCE) cells from low- and high-dose UVB damage. In this study, 1 μg/ml of $T\beta_4$ was added to BCE cells 2 h before low (12.5 mj/cm^2) or high dosage (100 mj/cm^2) UVB exposure. Using a fluorogenic substrate cleavage assay, we found that $T\beta_4$ diminished the reactive oxygen species level in BCE cells elicited by UVB. However, the protection of viability by $T\beta_4$ could only be detected under low-dose UVB exposure. Moreover, both caspase-9 activity and annexin V/propidium iodine staining demonstrated that $T\beta_4$ only protected BCE cells from low-dose UVB-induced apoptosis but not high-dose UVB-induced necrosis. Together, $T\beta_4$ protected corneal endothelial cells from UVB-induced oxidative stress and apoptosis after low-dose UVB exposure. The results support further investigation towards topical use or anterior chamber injection of this small hydrophilic peptide in treating and preventing UVB-induced corneal endothelial damage.

Key Words: thymosin β_4, corneal endothelial cells, ultraviolet B

Introduction

Solar ultraviolet rays (UVR) can be divided into UVA (315-400 nm), UVB (280-315 nm) and UVC (230-280 nm) according to their wavelengths. Cornea, the outermost tissue of eyeball, absorbs most of UVB by its epithelium and endothelium and protects the development of cataract and maculopathy (5, 18). Photokeratitis (29), corneal haze and edema (28) can all result from acute excessive UVB exposure.

Corneal endothelium plays a central role in maintaining corneal transparency by acting not only as a permeability barrier between the aqueous humor and the corneal stroma, but also as a pump to keep the cornea in a partially dehydrated state (16). Injury of corneal endothelium by UVB (20, 23) or other conditions such as surgical insult (12) or graft rejection (3) may result in its cell loss. Due to the limited proliferation capacity after birth, repair of corneal endothelium mainly relies on cell migration and rearrangement as well as on the enlargement of residual cells (15). Therefore, maintaining the viability of

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corneal endothelial cells and avoiding cell loss are crucial for corneal survival (3, 10). In contrast to a number of studies dedicated to the discovery the antidotes for UVB-induced damage on corneal epithelial cells (9, 17, 24, 28), much less effort has been made to identify the agents that protect corneal endothelial cells against similar insults (23).

Thymosin beta-4 ($T\beta_4$) has been reported to have anti-apoptotic effects on corneal epithelial cells (6, 27), conjunctival cells (26) and cardiomyocyte (1, 4). Sosne *et al*. reported that exogenous $T\beta_4$ inhibits ethanol (27) and benzalkonium chloride (26)-mediated apoptosis in corneal epithelial and conjunctival cells. Apoptosis, the ultimate pathological consequence of cornea epithelial (25) and endothelial (23) injuries induced by low-dose UVB, has been reported to increase intracellular oxidative stress by excessive formation of reactive oxygen species (ROS) (11, 14). Our previous experiments demonstrated that exogenous $T\beta_4$ protected human corneal epithelial cells against H_2O_2-triggered apoptosis (6) by diminishing intracellular oxidative stress *via* upregulation of anti-oxidative enzymes (7). However, the effect of $T\beta_4$ on UVB-induced damage is still unclear. The purpose of this study is to investigate the protective effects of $T\beta_4$ on corneal endothelial cells against different doses of UVB exposure.

Materials and Methods

Cell Culture

Bovine corneal endothelial (BCE) cells, purchased from American Type Culture Collection (ATCC, No. CRL-2048), were maintained in DMEM medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) in 95% air and 5% CO_2 at 37°C. Cells were subcultured every 3 days.

Preparation of Recombinant $T\beta_4$

Preparation of the histidine-tagged $T\beta_4$ fusion protein (His_6-$T\beta_4$) was done as described previously (6). The concentration of exogenous $T\beta_4$ used to treat cells was 1 mg/ml based on our previous apoptotic studies on corneal cells (6). The concentration of $T\beta_4$ from 0.5-2 μg/ml with exogenous administration showed dose-responsive anti-apoptotic effect.

UVB Irradiation

UVB exposure was conducted by placing BCE cells on a UV crosslinker (Model CL-1000, UVP Inc., Upland, CA, USA). The output energy was 100 mj/cm^2/sec and the total dosage was calculated according to the length of exposure. The lid of the culture plate was removed during UVB exposure.

Measurement of Intracellular ROS Levels

BCE cells were incubated with or without exogenous $T\beta_4$ for 2 h before being exposed to 12.5 and 100 mj/cm^2 of UVB, respectively. For measuring the level of intracellular ROS, cells were incubated with 5 mg/ml hydroethidine (21) (HE, Molecular Probes, Eugene, OR, USA) at 37°C in the dark for 30 min. Cells were then harvested and resuspended in 50 mM HEPES buffer (5 mM HEPES, pH 7.4; 5 mM KCl, 140 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM glucose) before their fluorescence intensities being analyzed by flow cytometry (FACS Calibur, BD Bioscience, San Jose, CA, USA).

Measurement of Cell Viability

BCE cells were seeded in 96-well plates (4×10^3 cells/well) the night before being treated without or with $T\beta_4$. Two hours later, cells were exposed to various dosages of UVB (0, 12.5, 25, 50, 100, 200, 400 mj/cm^2). After incubation for another 24 h, culture medium in each well was replaced by serum-free DMEM (100 ml) containing 20 μl MTS reagent (Promega, Medison, WI, USA) and the OD_{490} was measured by a microplate reader (Bio-Rad, Hercules, CA, USA) after cells were incubated in the dark at 37°C for another 1-4 h.

Detection of the Activation of Caspases-9 and Caspase-8

BCE cells were incubated with exogenous $T\beta_4$ for 2 h before exposure to 12.5 mj/cm^2 of UVB. For examining the *in situ* activation of caspases-9 and -8, a fluorogenic substrates cleavage assay (OncoImmunin, College Park, MD, USA) was performed as previously described (7). The fluorescence was detected by flow cytometry and the intensity of fluorescence was calculated using Modfit software (Verity Software House, Topsham, ME, USA). For analyzing the activity of caspase-9 in cell lysate, a colorimetric substrate cleavage assay (R&D Systems, Minneapolis, MN, USA) was performed as previously described (6).

Annexin V and Propidium Iodine (PI) Staining

BCE cells were seeded on cover slips placed in a 24-well plate (3×10^4 cells/well) a night before exposure to low- (12.5 mj/cm^2) or high-dose (100 mj/cm^2) of UVB. Twenty-four h later after two gentle PBS washes, cells were incubated in 200 ml of a buffer containing 5 ml of FITC-conjugated Annexin V (BD Bioscience) and 10 μl of PI (BD Bioscience) in the

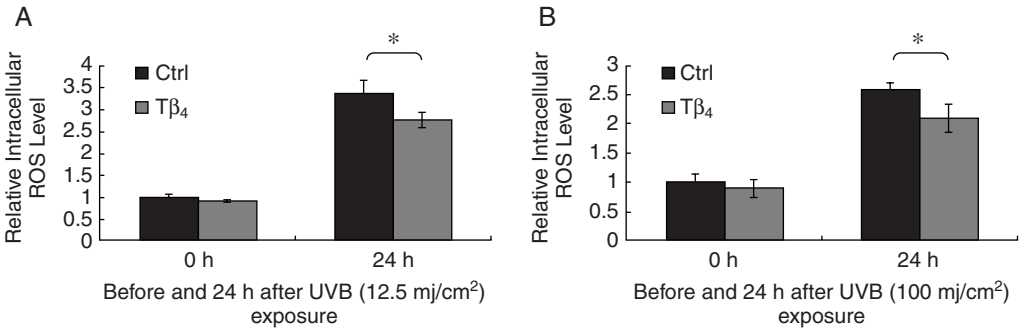

Fig. 1. Reducing UVB-induced intracellular oxidative stress in BCE cells by $T\beta_4$. $T\beta_4$ (1 μg/ml) was added to BCE cells 2 h before UVB exposure. Intracellular ROS level was measured by HE before and 24 h after exposed to (A) low dose (12.5 mj/cm^2) or (B) high dose (100 mj/cm^2) of UVB. UVB increased intracellular oxidative stress in BCE cells which could be reduced by pre-treatment of $T\beta_4$ (*t*-test, **P* < 0.05, n = 3).

dark at room temperature for 15 min. Following PBS washes, cells were fixed with 2% formaldehyde in the dark for 15 min before being mounted for fluorescence microscopy (Leitz, Wetzlar, Germany). Image analysis was performed with the SPOT RT Imaging system (Diagnostic Instruments, Sterling Heights, MI, USA).

Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Science-10 software (SPSS Inc., Chicago, IL, USA). Data from the caspase-9 colorimetric substrate cleavage assay were analyzed by ANOVA tests with Tukey's Post-Hoc tests at 95% confidence interval. Results from intracellular ROS assays, MTS assay and fluorogenic assays for caspase-9 and -8 activity were determined by two-tail, non-pair *t* tests, and *P* value less than 0.05 was considered as statistically significant.

Results

$T\beta_4$ Reduces Intracellular Oxidative Stress in Corneal Endothelial Cells Elicited by UVB

We first asked whether exogenous $T\beta_4$ could diminish UVB-induced intracellular oxidative stress. BCE cells were exposed to 12.5 mj/cm^2 (Fig. 1A) or 100 mj/cm^2 (Fig. 1B) UVB, respectively. It was found that $T\beta_4$ did not change the intracellular ROS level in BCE cells without UVB exposure. Both low (12.5 mj/cm^2) and high (100 mj/cm^2) doses of UVB exposure could significantly increase intracellular ROS levels 24 h post-injury, and the increase of ROS were partially but significantly reduced by $T\beta_4$.

$T\beta_4$ Protects Corneal Endothelial Cells against Low-Dose UVB-Induced Cell Death

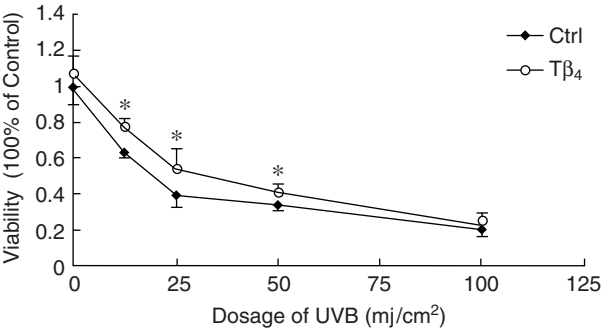

Fig. 2. Protection of BCE cells by $T\beta_4$ against low-dose of UVB-induced cell death. $T\beta_4$ (1 μg/ml) was added to BCE cells 2 h before UVB exposure. Cell viability was measured by MTS assay 24 h after various dosages of UVB exposure. $T\beta_4$ protected BCE cells from 12.5, 25 and 50 mj/cm^2 of UVB-induced damage but failed to protect cells from 100 mj/cm^2 of UVB-induced cell death. (*t*-test, **P* < 0.05, n = 3)

We next asked if $T\beta_4$-induced protection against UVB-triggered cell death. $T\beta_4$ was added to BCE cells 2 h before being exposed to various dosages of UVB. Twenty-four hours later, the viability of these cells was measured. It was found that $T\beta_4$ effectively ameliorated low-dose (12.5, 25, 50 mj/cm^2) UVB-induced death of BCE cells but did not protect BCE cells from high-dose (100 mj/cm^2) UVB-triggered death (Fig. 2) since the ROS level was reduced by $T\beta_4$ (Fig. 1B).

$T\beta_4$ Abrogates the Intrinsic Pathway-Mediated Apoptosis Triggered by Low-Dose UVB

The activities of caspases-9 and -8 in BCE cells 24 h post-UVB (12.5 mj/cm^2) exposure were subsequently measured by flow cytometric analyses. Significant activation of caspase-9 was detected but not for caspase-8 (Fig. 3A). $T\beta_4$ did not alter the

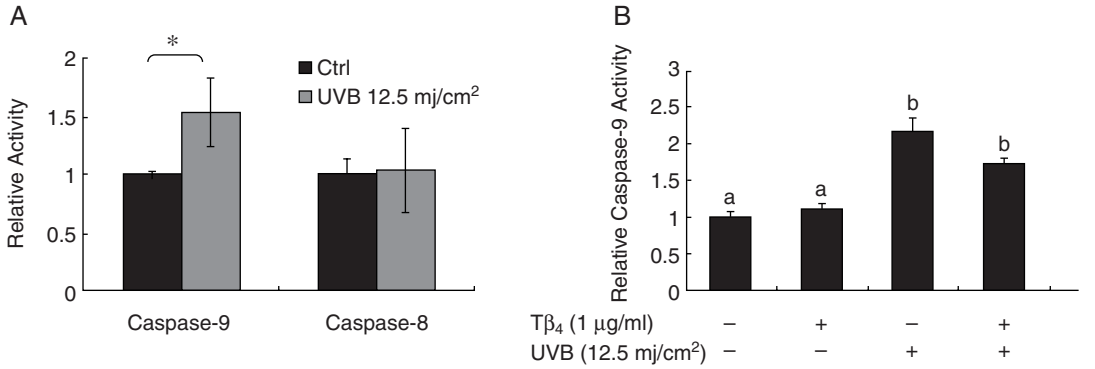

Fig. 3. Inhibition of UVB-triggered intrinsic pathway activation in BCE cells by Tβ4. (A) Fluorogenic substrate assay for detection of intracellular caspase-9 and -8 activities in BCE cells were performed before and 24 h after low dose (12.5 mj/cm^2) of UVB exposure. Only caspase-9 activity was increased by 12.5 mj/cm^2 UVB (*t*-test, **P* < 0.05, n = 3). (B) Caspsae-9 activity was measured by a colorimetric assay 24 h after low dose (12.5 mj/cm^2) of UVB exposure. Pre-treatment of Tβ4 did not alter caspase-9 activity in BCE cells but significantly reduced caspase-9 activity elicited by 12.5 mj/cm^2 UVB. Data were analyzed by ANOVA with Tukey Post-Hoc tests and different characters represent different levels of significance at 95% confidence intervals (n = 3).

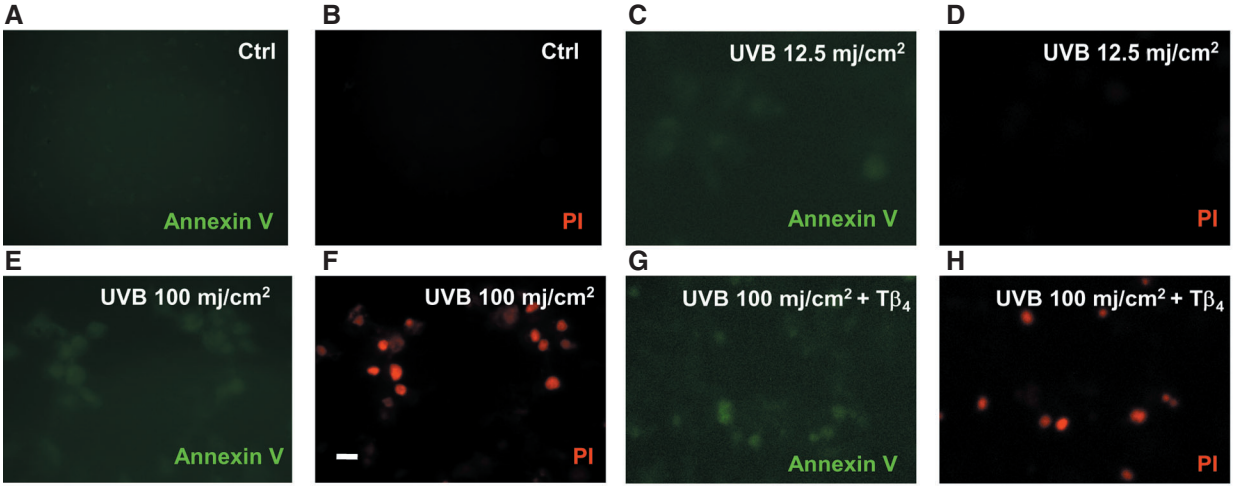

Fig. 4. Low dose of UVB results in apoptosis while high dose of UVB results in necrosis of BCE cells. Apoptosis versus necrosis was distinguished by co-staining annexin V with PI 24 h after BCE cells were exposed to UVB. When exposed to low dose (12.5 mj/cm^2) of UVB, cells showed weak positive of annexin V (C) but negative of PI (D) staining comparison of cells without UVB exposure (A, B). Weak positive of annexin V (E, G) and strong positive PI (F, H) staining could be detected in BCE cells without (E, F) or with (G, H) pretreatment of Tβ4 for 2 h when cells were exposed to high dose (100 mj/cm^2) of UVB. Bar, 50 μm.

activity of caspase-9 in BCE cells (Fig. 3B). However, Tβ4 abrogated the intrinsic pathway-mediated apoptosis evidenced by inhibited low-dose (12.5 mj/cm^2) UVB-triggered activation of caspase-9 (Fig. 3B).

Tβ4 did not Protect Corneal Endothelial Cells from High-Dose UVB-Induced Necrosis

To elucidate the pathomechanism of high-dose UVB-induced cell death, annexin V/PI staining was performed on BCE cells 24 h after UVB exposure. In the control group, cells were negative for both annexin V/PI staining without UVB exposure (Fig. 4, A and B). Cells were positive for annexin V staining after exposure to both low- (12.5 mj/cm^2) and high-dose (100 mj/cm^2) UVB (Figs. 4C and 4E, respectively). However, only high-dose (100 mj/cm^2) UVB on BCE cells showed positive for PI staining (Fig. 4F) while low-dose (12.5 mj/cm^2) UVB did not (Fig. 4D). Moreover, pretreatment of Tβ4 for 2 h did not show protection of BCE cells from high-dose (100 mj/cm^2) UVB-induced cell death (Figs. 4G and 4H). The results suggested that BCE cells primarily died of

apoptosis under low-dose UVB and died of necrosis under high-dose UVB.

Discussion

In the past decades, the painful cost for rapid economical development is the deterioration of our ecosystem (2). For this reason, an increased incidence of loss of vision from cataract and maculopathy has resulted from a higher fluency of UV light at the earth surface due to ozone depletion (30). While corneal epithelial cells are constantly being renewed due to the proliferation of limbal stem/progenitor cells (13), corneal endothelial cells are much more difficult to be replenished because of their proliferation potential. It is, therefore, imperative to develop corneal endothelium-protecting agents to reduce the incidence of irreversible UVB-induced corneal injury caused by UVB exposure.

$T\beta_4$ has previously been shown to protect corneal epithelial cells from apoptosis (6, 26, 27) and its internalization appears to be crucial for such effects (6). Internalized $T\beta_4$ upregulates several crucial antioxidative enzymes to reduce intracellular oxidative stress elicited by H_2O_2 and plays a key role in mechanisms of anti-intrinsic pathway-mediated apoptosis (7). In the current study, we found that $T\beta_4$ was also efficiently internalized into BCE cells within 2 h after exogenous administration (data not shown). UVB increased the ROS level in BCE cells (Figs. 1A and 1B). Exogenous $T\beta_4$ reduced intracellular oxidative stress elicited by both low (Fig. 1A) and high dose (Fig. 1B) of UVB irradiation. However, we found that cytoprotection of $T\beta_4$ on BCE cells were only against low-dose UVB injury whereas there was no cytoprotective effect against high-dose UVB damage (Fig. 2). When BCE cells were exposed to low-dose UVB, selective activation of caspase-9 (Fig. 3A) was noted and $T\beta_4$ possessed its anti-intrinsic pathway-mediated apoptosis by inhibiting the activation of caspase-9 (Fig. 3B) and decreasing intracellular ROS levels (Fig. 1A). In our previous study, up-regulation of manganese superoxide dismutase by $T\beta_4$ in corneal cells had been found to contribute to the anti-intrinsic pathway-mediated apoptosis (7). On the other hand, high dose of UVB irradiation on BCE cells triggered both apoptosis and necrosis (Figs. 4E and 4F) whereas low-dose UVB-induced apoptosis (Figs. 3A, 4C and 4D). $T\beta_4$ failed to rescue BCE cells from high dose of UVB (Figs. 2, 4G and 4H) suggesting its limited anti-necrosis ability.

It is known that corneal injuries induced by UVB are correlated with total cumulative dosage (20, 25). In this study, BCE cells underwent apoptosis under both low- and high-dose UVB but necrosis occurred only under high-dose UVB. $T\beta_4$ possessed anti-apoptotic but not anti-necrotic ability in spite of decreased ROS levels. Moreover, compared to the protection of H_2O_2-induced apoptosis that we had previously reported (6, 7), exogenous $T\beta_4$ only moderately protected BCE cells from UVB-induced apoptosis. A possible explanation for stronger protection of $T\beta_4$ against H_2O_2- and UVB-induced damage is the complexity of death signaling triggered by the latter. DNA photoproducts such as cyclobutane pyrimidine dimmers (19) and activated mitogen-activated protein kinase (22) are also involved in UVB-induced cell death. So far there is no evidence that $T\beta_4$ interferes with the above mentioned mechanisms. Besides, it was not clear whether this is due to inadequate concentration of $T\beta_4$. Future efforts will be made to examine whether higher concentrations of $T\beta_4$ confers better protection.

In summary, protection of corneal cells from UVB is imperative. Our results suggest that the exogenous $T\beta_4$ protects BCE cells against apoptosis induced by low-dose UVB but not necrosis triggered by high-dose UVB. Because of its small size, good water solubility (8), rapid cellular entry as well as its anti-apoptotic activities, $T\beta_4$ may be a good candidate for topical or anterior chamber administration to prevent and/or rescue the acute endothelial cell apoptosis triggered by UVB.

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