

Title page

Title:

Internalization is essential for the anti-apoptotic effect of exogenous thymosin beta-4 on human corneal epithelial cells

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Running head:

T β ₄ internalized critical for anti-apoptosis

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Abstract

Thymosin β -4 ($T\beta_4$) is an ubiquitous peptide consisting of 43 amino acids that has pleiotropic effects on different types of cells. In addition to preventing the death of cardiomyocytes induced by ischemia, exogenous $T\beta_4$ has been shown to inhibit the apoptosis of human corneal epithelial cells triggered by ethanol. In the present study, we found that pretreatment of the immortalized human corneal epithelial (HCE-T) cells with the recombinant $T\beta_4$ produced by *E. coli* reduced their sensitivity to FasL and H_2O_2 . Moreover, activation of caspases-8 and -3 by FasL as well as that of caspases-9 and -3 by H_2O_2 in these cells was also abolished by $T\beta_4$ pretreatment. Interestingly, internalization of this G-actin sequestering peptide into HCE-T cells was demonstrated by immunofluorescence staining. Surprisingly, not only was the internalization of $T\beta_4$ but also its anti-apoptotic effect abrogated when HCE-T cells were incubated with cytochalasin D, an inhibitor of endocytosis, prior to the addition of exogenous $T\beta_4$. To our best knowledge, this is the first report to show that cellular uptake is essential for the exogenous $T\beta_4$ in protecting human corneal epithelial cells from FasL- and H_2O_2 -induced apoptosis.

Introduction

Apoptosis is the pathomechanism of many corneal diseases. Signaling through death receptors and/ or mitochondrial activation are the major pathways responsible for apoptosis (Green DR et al. 1998; Sun XM et al. 1999).

Thymosin β -4 ($T\beta_4$) is a ubiquitous, abundant intracellular peptide consisting of 43-amino acids which regulates the actin monomer pool by sequestering G-actin (Huff T et al., 2001). $T\beta_4$ has pleiotropic effects on different types of cells. In corneal epithelial cells, wound healing promotion, inflammation reduction (Sosne G et al., 2002; Sosne G et al., 2005), and inhibition of ethanol-induced apoptosis (Sosne G et al., 2004) by exogenous $T\beta_4$ have been reported. However, the precise protective mechanisms of this G-actin sequestering peptide on corneal epithelial cells against various injuries are not fully understood.

The purpose of this study is to verify the anti-apoptotic effect of exogenous $T\beta_4$ on human corneal epithelial cells and dissect subsequently its underlying mechanism. We showed that exogenous $T\beta_4$ not only prevents the death of the immortalized human corneal epithelial (HCE-T) cells induced by FasL and H_2O_2 but inhibits the activation of several key caspases. Furthermore, internalization of $T\beta_4$ was demonstrated by immunofluorescence staining which, to our surprise, is essential for its protection on HCE-T cells against the cytotoxicity of both FasL and H_2O_2 .

Materials and Methods

Cell lines and culturing: SV-40 immortalized HCE-T cells were kindly given from Dr. Araki-Sasaki K (RIKEN BioResource Center, Ibaraki Japan). **Exogenous T β ₄:** To generate the histidine-tagged T β ₄ fusion protein, cDNA sequence encoding full length mouse T β ₄ was amplified by PCR and inserted into the BamHI/EcoR1 site of pET11d vector (Navagen, Darmstadt Germany).

MTS assay: HCE-T cells were seeded in 96-well plate (4000 cells/well) overnight and cells were incubated with various concentration of H₂O₂ for 24 hours. Sucking out all culture medium before 20 μ l MTS reagent (Promega, WI USA) plus 100 μ l DMEM was added in each well of 96 plate. After incubation in dark at 37 °C for 1-4 hours, measure absorption at 490 nm.

Flow cytometry: HCE-T cells were incubated with 1 μ g/ml of exogenous T β ₄ for 24 hours. Cells were detached and stained with FITC-coupled mouse antibody against human CD95 (BD Bioscience, CA USA) and analyzed with flow cytometry.

Immunofluorescence stain for exogenous T β ₄: HCE-T cells were incubated with histidine-tagged T β ₄ for 1, 2, 18 hours following stained for intracellular proteins, or incubated with histidine-tagged T β ₄ for 2 hours and stained for intracellular protein after exogenous T β ₄ washed out for 24 hours.

Drug treatment: HCE-T cells were treated with cytochalasin D in concentration of 20 mM (Sigma-Aldrich, MO USA) for 30 minutes to detect intracellular caspase activities and 1 hour to immunostain prior to incubate with exogenous T β ₄.

Immunofluorescence stain for caspase-3 activity: HCE-T cells were incubated with fluorogenic caspase-3 substrate (OncoImmunin, MD USA) in 37°C for 1 hour. **Caspase colorimetric assay:** The supernatant reacted with substrates of caspase-3,-8 or-9 activity (R & D system, MN USA) in 37°C for 1 to 2 hours. Absorbance was read with microplate reader (Bio-Rad, CA USA) at 405 nm.

Results

Preparation of the recombinant T β ₄ and the anti-T β ₄ polyclonal antibody

To determine the purity of the recombinant histidine-tagged T β ₄ (His-T β ₄), SDS-PAGE was performed using a synthetic T β ₄ as a positive control. As shown in Fig. 1A, the recombinant T β ₄ exhibited a single band with molecular weight similar to that of the synthetic peptide. Western blotting was then conducted to assess the usefulness of the polyclonal anti-T β ₄ antibody generated by us. Indeed, our antibody recognized both the recombinant and the synthetic peptides (Fig. 1B, lanes 1 and 2). By contrast, only the recombinant T β ₄ could be detected by the anti-His antibody (Fig. 1B, lanes 3 and 4).

Exogenous T β ₄ protects HCE-T cells against FasL- and H₂O₂-induced injury

To examine the anti-apoptotic effect of exogenous T β ₄ on human corneal epithelial (HCE-T) cells, we first assessed whether these cells could be killed by FasL and H₂O₂. Surface expression of Fas (CD95) on HCE-T cells was demonstrated by flow cytometric analysis (Fig. 2A, B) and upon FasL (anti-Fas IgM, clone 11; 40 ng/ml) treatment, these cells underwent apoptosis indicated by cellular shrinkage, vacuolated cytoplasm, and nuclear condensation (Figure 2E). On the other hand, most of the HCE-T cells looked healthy when recombinant T β ₄ (1 μ g/ml) was added before FasL treatment (Fig. 2F). The protective effects of exogenous T β ₄ on HCE-T cells against FasL were then analyzed by both the MTT (for viability) and substrate cleavage (for caspases-3 and -8 activities) assays. Indeed, pretreating these cells with exogenous T β ₄ for 2 hours markedly increased their survival (Fig. 2G) and in the meantime, decreased FasL-triggered activation of both caspase-8 (Fig. 2H) and caspase-3 (Fig. 2I). Varying concentrations of H₂O₂ (100, 200, 400, 600 and 800 μ M) were then used to determine the lethal concentration (LC) for HCE-T cells and its LC₅₀ was between

Discussion

Corneal epithelium covers on the surface cornea as the first defense of eyeball against exogenous pathogens, daily UV damage, chemical injury, and is sensitive to tear film insufficiency, intraocular pressure (IOP) elevation, contact lens induced hypoxic status (Kurpakus-Wheater M et al., 2003). Parts of corneal epithelial cells undergo apoptosis and shed every day due to their finite lifespan. Either disease or stress accelerates corneal epithelial cell apoptosis that results in corneal erosion (Vemuganti GK et al., 2004). There are two major apoptotic pathways. Signaling through death receptors activates extrinsic pathway and mitochondrial activation is intrinsic pathway. However, in most condition of corneal injury, it is hard to say that only single apoptotic pathway specific responses to one injury due to interaction between extrinsic and intrinsic pathway (Chandra J et al. Free Radical Bio Med 2000; Szentmary N et al., 2005 $T\beta_4$, a G-actin sequestering peptide, promotes wound healing, decreases inflammation and inhibits ethanol-induced apoptosis. The multiple protect effects encourage therapeutic use of exogenous $T\beta_4$.

The anti-apoptotic effect of exogenous $T\beta_4$ has been reported. Sosne G reported that exogenous $T\beta_4$ inhibited intracellular caspase-2, -3, -8 and -9 activity, decreased cytochrome c release, and increased Bcl-2 expression in ethanol-exposed human corneal epithelial cells (Sosne G et al., 2004). Bock-Marquette found a marked decrease in cell death by TUNEL assay in $T\beta_4$ -treated cardiomyocytes 24 hour after coronary artery ligation in mice (Bock-Marquette I et al., 2004). We assume that the anti-apoptotic effect of $T\beta_4$, a small molecule with multiple biological functions, should not be constricted in abrogating either intrinsic or extrinsic pathway. To prove our hypothesis, we used FasL-mediated cytotoxicity as the extrinsic apoptosis, and H_2O_2 as the intrinsic apoptosis. Not beyond expectation, the protect effect of

Figure 1

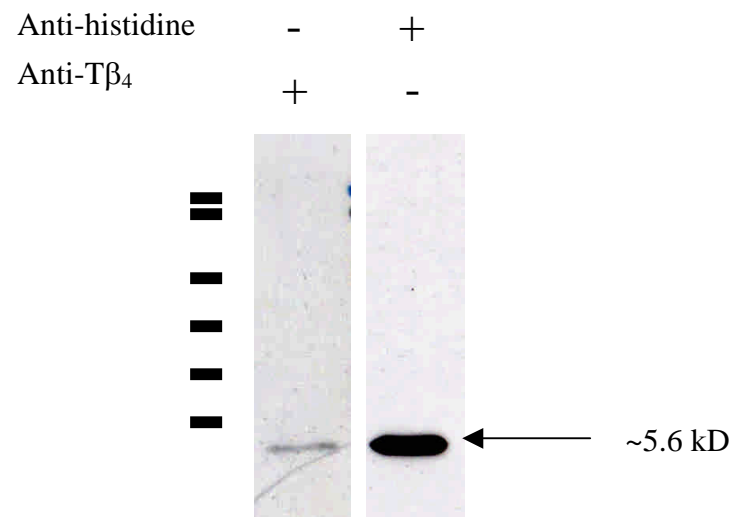


Figure 2

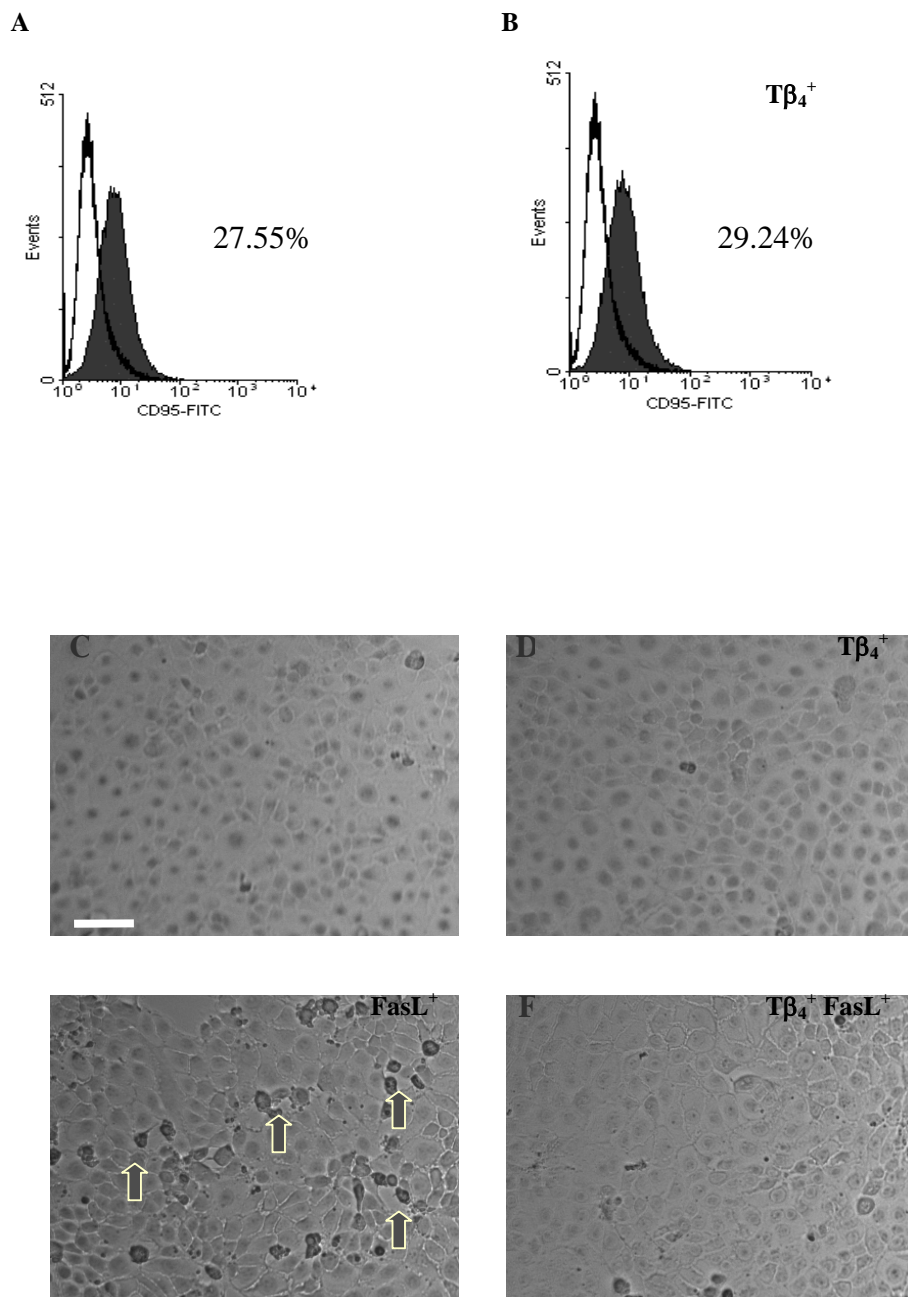
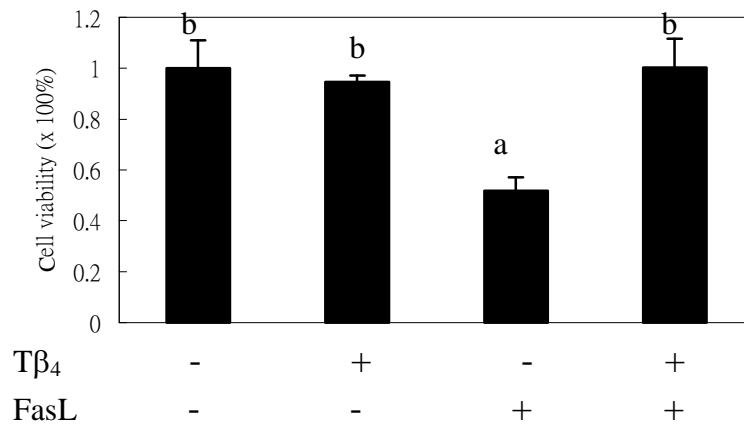
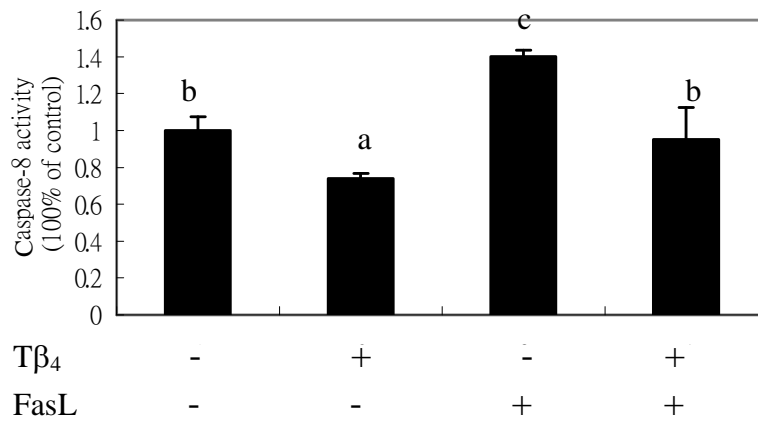


Figure 2

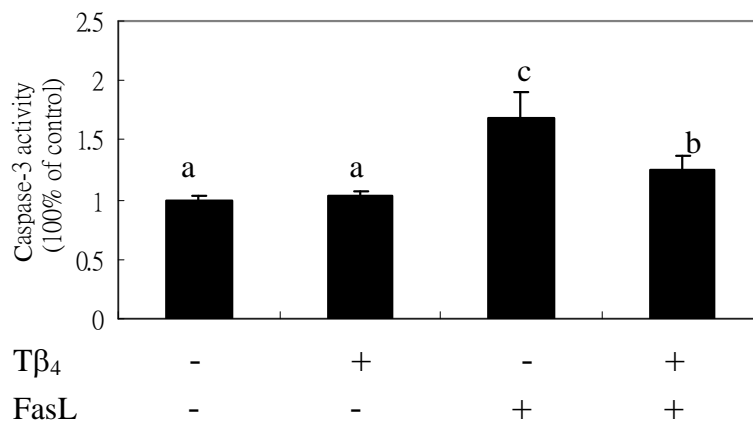
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Legends

Figure 1: Western blot of recombinant histidine-tagged T β ₄ peptide. E.coli produced T β ₄ peptide with six histidines at the N-terminal showed the molecular weight at 5.6 kD.

Figure 2: Exogenous T β ₄ protected HCE-T cells against FasL-induced Apoptosis.

(2A) Surface expression of CD95 in HCE-T cells was detected by flow cytometry. 27.55% of HCE-T cells expressed CD95 antigen on cell surface. **(2B)** Neither up-regulated nor down-regulated CD95 expression of HCE-T cells was noted after incubation with T β ₄ for 24 hours. **(2C)** HCE-T cells. **(2D)** Exogenous T β ₄ was added to HCE-T cells for 2 hours. **(2E)** HCE-T cells were incubated with FasL (40 ng/ml) for 18 hours. The morphology of apoptotic HCE-T cells characterized as nucleus condensation with intense chromatin around the nuclear periphery, shrunken cells with vacuolated cytoplasm. **(2F)** Exogenous T β ₄ was added to HCE-T cells 2 hours before FasL. Most of HCE-T cells were healthy and kept cobble stone-like appearance. (bar scale = 2 μ m) **(2G)** MTT assay of HCE-T cells were performed after continuous FasL injury for 24 hours. Without exogenous T β ₄, FasL injury result in marked decreased in cell viability. However, added exogenous T β ₄ for 2 hours prior to FasL injury, cell viability didn't show significant difference from that without FasL injury. (ANOVA, Tukey Post Hoc Test, p<.05, n=3) **(2H, I)** HCE-T cells were incubated with FasL for 24 hours and intracellular caspase-8 **(2H)**, -3 **(2I)** were detected by colorimetric assay. Exogenous T β ₄ decreased intracellular caspase-8, -3 activities when HCE-T cells suffered from apoptosis, and decreased baseline intracellular caspase-8 activities, but not caspase-3 activity. (ANOVA, Tukey Post Hoc Test, p<.05, n=3)

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