Thymosin beta 4: A novel corneal wound healing and anti-inflammatory agent

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Abstract: Thymosin beta 4 (Tβ_4) is a low molecular weight protein present in all cells except erythrocytes. Although Tβ_4 is the major monomeric actin-sequestering peptide in cells and can depolymerize F-actin, evidence is mounting to support the idea that it has multiple, seemingly diverse, cellular functions. In cornea, as in other tissues, Tβ_4 promotes cell migration and wound healing, has anti-inflammatory properties, and suppresses apoptosis. In this review we discuss the current state of knowledge regarding the effects of Tβ_4 in maintaining the healthy, functional cornea. The clinical implications of the use of Tβ_4 as a wound healing and anti-inflammatory agent are discussed.

Keywords: apoptosis, cell migration, cornea, inflammation, thymosin beta 4, wound healing

Corneal epithelial wounds: overview

The cornea consists of three types of cells, epithelial, stromal, and endothelial. It is avascular, transparent, and the most sensitive tissue in the body. The cornea functions as an optical lens that refracts light, but it also contributes to the rigid shell of the eyeball. In order to optimally refract light and ensure clear vision, the cornea must maintain optical transparency. This is accomplished in the stroma by a well-organized arrangement of collagen fibers into lamellae (Meek et al 2003). The endothelium functions to preserve corneal transparency by regulating the level of hydration in the stroma. The corneal epithelium also contributes to the maintenance of corneal transparency (Verkman 2003). Damage to the corneal epithelium caused by chemical, physical or microbial insults can lead to swelling of the stroma, the activation of stromal fibroblasts, and the infiltration of inflammatory cells, leading to the loss of corneal transparency and integrity. Timely resurfacing of the epithelium after injury is thus essential in preventing the loss of normal corneal function and loss of vision.

Following wounding, corneal epithelial healing occurs in several stages by a combination of three separate mechanisms, migration, mitosis, and differentiation (Gipson and Inatomi 1995). Through a highly organized chain of events, cells must migrate to cover the denuded stroma, and then proliferate and differentiate to restore the normal multi-layered epithelial cytoarchitecture (Zhao et al 2003). In most instances, corneal epithelial defects caused by a simple injury, such as a surface scrape or abrasion, are healed promptly. However, in individuals with certain clinical disorders, such as diabetic keratopathy, basement membrane dystrophies, neuropathies, and infections, corneal epithelial defects persist and do not respond to conventional treatment regimens (Sanchez-Thorin 1998). Not only is the rate of corneal re-epithelialization important after injury, the re-normalization of the epithelial cytoarchitecture and re-establishment of the epithelial barrier are paramount (Huang et al 1990).

In order to re-establish the epithelial barrier, cells migrate over the denuded surface of the cornea in a manner that is dependent both on the interaction of the cells with the underlying substrate via cell-extracellular matrix adhesions called hemidesmosomes,

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Table 1 Properties of thymosin beta 4 in the eye

- Stimulates corneal and conjunctival epithelial cell migration in vitro
- Modulates production of metalloproteinases (MMP-1, -2, and -9)
- Accelerates corneal re-epithelialization in vivo after alkali injury and scrape wounding
- Inhibits corneal neutrophil infiltration after alkali injury in vivo
- Modulates and down-regulates a number of key inflammatory cytokines (IL-1β, TNF-α) and chemokines (MIP-1α and β, MIP-2, MCP-1) in the cornea
- Inhibits TNF-α-stimulated NFκB activation and nuclear translocation in corneal epithelial cells
- Increases TGF-β1 and laminin-5 expression in corneal epithelial cells
- Inhibits corneal epithelial cell apoptosis

and on cell–cell adhesions called desmosomes (Suzuki et al 2003). Regeneration of cell adhesions to reconstitute the epithelial barrier protects the cornea from infectious agents and also is important to prevent the persistence of corneal epithelial fragility that can lead to recurrent erosions (McCartney and Cantu-Crouch 1992).

The wound repair process is intricately linked to a complex inflammatory response that must be precisely regulated to ensure proper healing and optimal visual outcome. Infiltration of inflammatory cells into injured corneal tissue is a hallmark of wound repair (Kenyon 1985). Retardation of epithelial recovery by persistent inflammation, release of enzymatic products from resident corneal cells and infiltrating inflammatory cells, and stimulation by cytokines and chemokines, all contribute to poor re-epithelialization. With regard to the cornea, detailed information on cytokine expression and activity is emerging, and great interest centers around identifying which cytokines and chemokines are induced in the cornea by a particular stimulant or insult (Wakefield and Lloyd 1992; Cubitt et al 1993, 1995).

Although much progress has been made in understanding how the cornea heals, current pharmacological therapies that accelerate and/or promote epithelial healing are severely limited. Clinicians still mainly aim to provide the patient with an environment conducive to healing and rely on the eye's innate ability to repair itself. Emerging evidence from our laboratory and others shows that thymosin beta 4 (Tβ_4) is a novel wound healing agent that promotes corneal repair and decreases inflammation after injury. In this review, we focus on the studies that provide evidence for the potential clinical usage of Tβ_4 as a corneal wound healing agent.

Properties of Tβ_4

Tβ_4 is a 43-amino acid, 4.9 kDa protein originally isolated from bovine thymus (Low et al 1981; Goodall et al 1983; Yu

et al 1994). Tβ_4 is the major monomeric actin-sequestering peptide in cells and can depolymerize F-actin. For example, it has been found to depolymerize cystic fibrosis-derived sputum actin in both a dose-dependent and a time-dependent manner (Rubin et al 2006). Tβ_4 influences T cell differentiation (Hu et al 1981; Low and Goldstein 1984). It has no signal sequence (Gondo et al 1987) but is found in serum (Naylor et al 1984; Weller et al 1988). Tβ_4 does not appear to bind to either heparin or other extracellular matrix molecules, suggesting it can rapidly diffuse throughout tissue compartments.

Tβ_4 is an ubiquitous polypeptide, highly conserved across species, and is found at concentrations of 1×10^{-5} to 5.6×10^{-1} M in a variety of tissues and cell types, yet, no receptors for the protein have been identified (Hannappel et al 1982; Hannappel and Leibold 1985; Hannappel and van Kampen 1987). A recent study suggests that internalization of exogenous Tβ_4 is essential for its subsequent cellular functions (Ho et al 2007).

Previous work identified Tβ_4 as an induced gene in endothelial cells undergoing differentiation in vitro (Grant et al 1995). Tβ_4 is active for endothelial cell and keratinocyte migration in vitro and for angiogenesis in vivo (Malinda et al 1997; Malinda et al 1999). Tβ_4 levels are highest in platelets and polymorphonuclear neutrophilic leukocytes (PMN), which are among the first formed elements and cells, respectively, to enter a wound and release their factors, some of which recruit additional cells to the wound site (Cassimeris et al 1992). Although the mechanism(s) of action of exogenous Tβ_4 on wound repair remains unclear, the high levels of Tβ_4 present in human wound fluid (13 µg/ml) suggest its importance in wound healing (Frohm et al 1996). Tβ_4 promotes cardiomyocyte migration, survival, and repair after experimental myocardial infarction, suggesting its potential as a new therapeutic target in the setting of acute myocardial damage (Bock-Marquette et al 2004). It is interesting to note

Table 2 Thymosin β_4: Some potential clinical ocular applications

- Inflammatory
  - Dry Eyes, Uveitis, Corneal Melts
- Infectious Keratitis (Bacterial, Viral and Fungal)
- Metabolic
  - Diabetic Eye Disease (Keratopathy)
- Trauma (chemical injury)
- Post Operative: cataract surgery, LASIK (Epi-LASIK, LASEK), PRK
- Other: Corneal transplants (PKP), Neurotrophic ulcers, Persistent epithelial defects, Recurrent erosions, Corneal abrasions

that Tβ$_4$ does not have any effect on cell proliferation (Leeanansaksiri et al 2004; Huang et al 2006; Sosne et al 2006).

In addition to its effects on wound healing, the anti-inflammatory properties of Tβ$_4$ are beginning to be elucidated. Young et al (1999) reported that Tβ$_4$ inhibited PMN chemotaxis to the bacterial chemoattractant, N-formyl-methionyl-leucyl-phenylalanine (fMLP). Whether Tβ$_4$ exerts its effects on PMN directly or indirectly is an intriguing question. Other studies showed that the activation-responsive expression of the lymph-specific form of Tβ$_4$ may be one mechanism by which dendritic epidermal T cells, and possibly other intraepithelial lymphocytes, down-regulate local inflammation (Girardi et al 2003). In a separate study, Tβ$_4$ lowered circulating levels of inflammatory cytokines and intermediates following LPS administration in vivo (Badamchian et al 2003). Additionally, Tβ$_4$ levels rapidly disappeared in the blood following LPS administration or during septic shock, suggesting that it may be involved in early events leading to activation of the inflammatory cascade and ultimately the clinical sequelae of sepsis.

Tβ$_4$ stimulates corneal epithelial cell migration and wound healing

Two major epithelial cell responses to wounding are cell migration to cover the wound bed, and cell proliferation to re-epithelialize the wound. We and others have shown that while not affecting cell proliferation, Tβ$_4$ significantly enhances the migration of endothelial cells, keratinocytes, tumor cells, and corneal epithelial cells (Sosne et al 2001, 2007; Moon et al 2006; Philp et al 2006). It has been proposed that Tβ$_4$ may promote migration via its upregulation of zyxin expression, a protein that regulates the actin cytoskeleton (Moon et al 2006). Tβ$_4$ promoted full thickness dermal wound repair in normal, steroid-treated and diabetic animal models (Malinda et al 1999; Philp et al 2003). These findings suggest that Tβ$_4$ may be useful in promoting corneal wound healing in healthy individuals as well as in people with healing-impaired pathologies like diabetes.

Because diabetic retinopathy (DR) corneas have delayed wound healing (Kabosova et al 2003), we investigated the expression of Tβ$_4$, a potent epithelial cell migration stimulator, in DR corneas. Human DR corneas were analyzed by gene microarray and quantitative RT-PCR and were found to express significantly less Tβ$_4$ compared to normal corneas (Saghizadeh et al 2006). We hypothesize that reduced expression of Tβ$_4$ may contribute to delayed wound healing in DR corneas. These findings may have important clinical relevance in the treatment of complications stemming from diabetic keratopathy.

In addition to systemic diseases that manifest in the cornea such as diabetic keratopathy, other commonly seen corneal wounds can range from simple epithelial scrape wounds, recurrent erosions, persistent epitheliopathies post-refractive surgery, to severe inflammatory reactions such as seen in alkali injury. Tβ$_4$ accelerated the re-epithelialization of corneal wounds in both heptanol debridement and alkali-burn animal models (Sosne et al 2001, 2002). Corneal alkali injury is marked by a massive
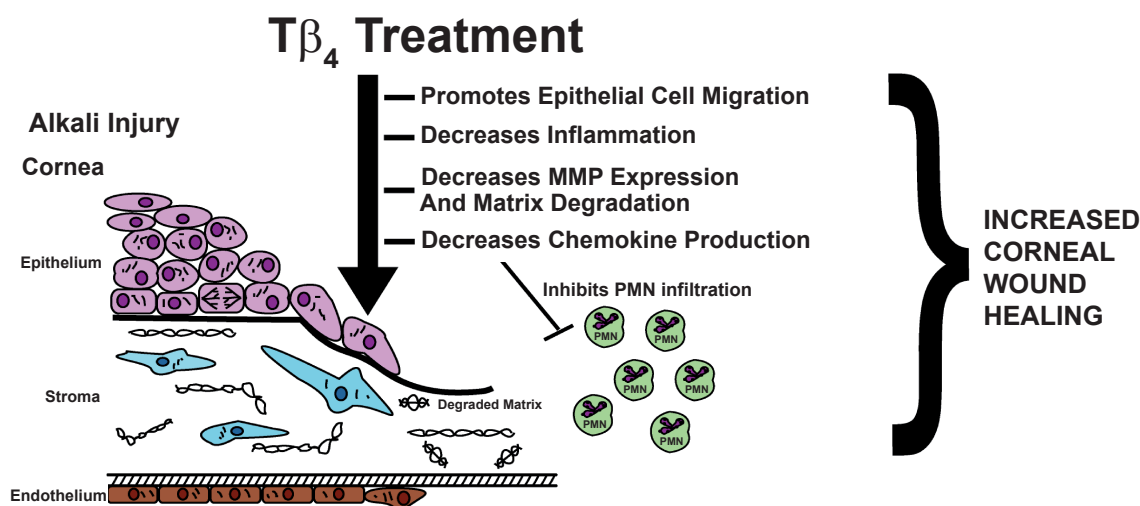

Figure 1 Tβ$_4$ promotes corneal wound healing. By modulating the corneal inflammatory response and promoting re-epithelialization in response to injury, Tβ$_4$ may be a new a therapeutic option for ophthalmologists treating corneal inflammatory and wound healing disorders.

PMN infiltration and inflammatory response characterized by over-expression of pro-inflammatory cytokines/chemokines and matrix-metalloproteinases (MMPs), much more extreme than that seen in the setting of scrape injury (Wagoner, 1997; Kenyon et al 1979; Foster et al 1982). The mouse alkali burn model mimics many aspects of the injury seen in humans by clinical slit-lamp and histopathological criteria. Slit-lamp observations of mouse eyes with alkali injury showed that 70% had readily apparent hyphema and total corneal opacification. In contrast 20% of alkali-injured eyes treated with $T\beta_4$ exhibited the same characteristics. The overall anatomical integrity of the anterior segment in the $T\beta_4$-treated eyes was markedly more normal in appearance, providing evidence that $T\beta_4$ treatment improves corneal healing and clarity following alkali injury (Sosne et al 2005).

Corneal epithelial wounds cannot heal properly without the re-establishment of cell attachment to the underlying connective tissue ie, basement membrane. Hemidesmosomes, specialized adhesion junctions present along the basal cell membrane of the basal cells of stratified squamous epithelia, are involved in the adherence of the basal cells to the basement membrane (Susi et al 1967; Jones et al 1994). Heptanol or alkali-burned corneal epithelial cells treated with $T\beta_4$ showed more hemidesmosomal adhesions to the basement membrane, and less widening of the space between epithelium and basement membrane. Laminin-5 is a major glycoprotein of the basement membrane underlying corneal epithelial cells. It plays a central role in both epithelial cell migration and in adhesion, and is associated with hemidesmosomes (Larjava et al 1993). In cultured corneal epithelial cells and in mouse cornea wounded by scraping, topical $T\beta_4$ treatment resulted in increased laminin-5 expression (Sosne et al 2002). This property of $T\beta_4$ has important clinical implications, since it could be utilized in the treatment of recurrent erosions or other situations in which corneal epithelial cell adhesion to the basement membrane is impaired.

As an essential step in the cascade of cellular events culminating in wound healing, epithelial cells participate in extracellular matrix synthesis and remodeling (Lu et al 2001; Santoro and Gaudino 2005). Human MMPs are a family of more than 20 zinc-dependent endopeptidases that upon activation degrade both matrix and non-matrix proteins as part of key rate-limiting steps in tissue repair and remodeling (Nagase et al 2006). $T\beta_4$ treatment modulates MMP expression in several epithelial models (Sosne et al 2005; 2007; Philp et al 2006). The majority of MMPs is secreted from the cell as non-catalytic zymogens (pro-MMPs) and is activated via removal of the inhibitory propeptides by proteinases such as thrombin, plasmin, trypsin, and other activated MMPs. Studies suggest that part of the wound healing activity of $T\beta_4$ may be related to its ability to transiently increase MMP activity (MMP-1, -2, and -9) via its central actin-binding domain (Philp et al 2006).

Using an in vitro model of corneal epithelial cell scrape wound healing we showed that blocking MMP activity with specific or broad-spectrum inhibitors decreased $T\beta_4$-mediated epithelial cell migration. This suggests that MMP catalytic activity is necessary for $T\beta_4$ promotion of epithelial cell migration (Sosne et al 2007). Alterations in MMP expression are linked to improper corneal wound healing and the formation of persistent epithelial defects and chronic corneal ulcers (Fini et al 1996). $T\beta_4$ differentially regulates the expression of MMPs in many tissues, and also regulates the MMP/TIMP balance in the cornea. Thus, it is plausible to hypothesize that $T\beta_4$ may promote corneal repair and matrix remodeling following wounding by modulating MMP expression and activity.

$T\beta_4$ modulates corneal inflammation

Many studies have contributed information regarding the roles of cytokine and chemokine expression and activity in the post-wound corneal inflammatory response in a wide array of clinical pathologies (Gillitzer and Goebeler 2001; Wilson et al 2003; Agrawal and Tsai 2003; Stramer et al 2004). For example, in chemically injured corneal epithelial cells, the levels of pro-inflammatory cytokines and chemokines are upregulated (Planck et al 1997; Sotozono et al 1997; Sosne et al 2002), and dry eye stimulates the expression of tumour necrosis factor-alpha (TNF-α) in mice (Luo et al 2004). Corneal epithelial monolayers infected with *Pseudomonas aeruginosa* demonstrated increased expression and secretion of IL-6, IL-8, and TNF-α (Zhang et al 2005). IL-6 has been associated with enhanced corneal epithelial cell migration (Wang et al 1994; Nakamura and Nishida 1999).

In the first ocular studies to provide evidence that $T\beta_4$-treatment accelerates *in vivo* wound healing and modulates corneal cytokine production, we showed in the rat corneal epithelial debridement model that mRNA transcripts for IL-1β and IL-6 were increased in $T\beta_4$-treated corneas after injury. Additionally, topical $T\beta_4$ treatment after alkali injury down-regulated the expression of the potent PMN chemoattractants, MIP-2 and KC, in the murine cornea. We suggested

that these cytokines may be involved in corneal inflammation and wound healing, and that the decreased corneal chemokine expression may be responsible for the observed decreased PMN infiltration.

TNF-alpha is a potent pro-inflammatory mediator that influences wound healing and apoptotic cell death (Zhang et al 2004). TNF-α stimulation activates transcription factors including nuclear factor kappa B (NFκB) (Baud and Karin 2001; Hanada and Yoshimura 2002; Ritchie et al 2004). NFκB dimers are maintained in an inactivated state in the cytoplasm by a family of inhibitory proteins, the IκBs. IκB can be phosphorylated following cellular stimulation, resulting in its degradation and release from the NFκB dimer. Activatied NFκB translocates to the nucleus where it binds to κB enhancer elements of a wide panel of target genes (Karin and Ben-Neriah 2000). Because of its ability to regulate the expression of inflammatory genes, NFκB is believed to play a major role in the inflammatory process (Hayden and Ghosh 2004).

In cultured human corneal epithelial cells stimulated with TNF-α, Tβ_4 treatment significantly decreased nuclear NFκB protein levels, NFκB activity, and p65 subunit phosphorylation. The nuclear translocation of NFκB was also inhibited. Although there are many inflammatory signaling pathways, we hypothesize that Tβ_4 mediates NFκB inflammatory signaling pathways in the cornea based on our findings. These observations have important clinical implications for the potential role of Tβ_4 as a corneal anti-inflammatory agent.

Tβ_4 inhibits corneal epithelial cell apoptosis

Evidence that Tβ_4 functions as an anti-apoptotic agent is mounting. HeLa cells that overexpressed Tβ_4 showed a higher growth rate and a lower percentage of basal apoptosis compared to control HeLa cells. In addition, the overexpressing cells were more resistant to paclitaxel-induced cell death (Oh et al 2006). The death of human corneal epithelial cells (HCET) induced by FasL or by hydrogen peroxide was significantly inhibited by Tβ_4 treatment. In this study it was observed that the internalization of exogenous Tβ_4 was essential for its anti-apoptotic activity in human corneal epithelial cells (Ho et al 2007). Resistance to FasL-induced apoptosis was also achieved in SW480 colon carcinoma cells that overexpressed Tβ_4 (Hsiao et al 2006). Using an *in vitro* culture model, we demonstrated that Tβ_4 suppressed ethanol-induced corneal epithelial cell apoptosis via the inhibition of caspases and suppression

of bcl-2 release from mitochondria (Sosne et al 2004). In cultured corneal and conjunctival epithelium, Tβ_4 did not protect cells from benzalkonium chloride-induced inhibition of proliferation, but apoptosis was significantly inhibited (Sosne et al 2006).

Regarding the mechanism by which Tβ_4 exerts its anti-apoptotic activity, it has been suggested that the reduction of early cell death initiation signals, such as the phosphorylation of c-Jun, may prevent apoptosis of neurons (Choi et al 2006). Additionally, it has been demonstrated that Tβ_4 promotes survival of cardiomyocytes through the formation of a functional complex with PINCH and integrin-linked kinase, resulting in activation of the survival kinase Akt (Bock-Marquette et al 2004).

Future directions

The mounting evidence suggesting the use of Tβ_4 as a novel corneal wound healing and anti-inflammatory agent presents an exciting new potential therapy for practicing ophthalmologists. The plethora of clinical entities related to retarded corneal wound healing present significant challenges for the practicing ophthalmologist. Tβ_4, a ubiquitous, naturally occurring molecule, has been demonstrated to promote corneal re-epithelialization, reduce inflammation and inhibit apoptosis. From routine corneal injuries such as abrasions and recurrent erosions to more severe inflammatory-mediated pathologies, ophthalmologists need an agent that can quickly promote corneal healing and properly modulate the inflammatory response.

More basic science studies focused on the mechanisms of action of Tβ_4 are needed to help guide future clinical applications. For example, it is interesting to speculate whether the anti-apoptotic properties of Tβ_4 could help to preserve tissue after acute injury or for corneal transplantation. Additionally, Tβ_4 may be useful as an adjuvant therapy to BAK-containing eye drops to prevent the adverse ocular surface side effects seen with many anti-glaucoma medications. Regarding the anti-inflammatory properties of Tβ_4, a wide array of clinical entities such as inflammatory keratopathies, corneal graft rejection, infections, uveitis, and dry eye syndromes may be just a few examples of potential therapeutic treatments. In addition to the basic science studies, we believe that the published animal studies showing Tβ_4's efficacy in a variety of models of injury provide a solid basis for testing the efficacy and safety of Tβ_4 in well controlled clinical trials and this is presently being planned (http://www.regenerx.com).

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