Animal studies with thymosin $\beta_4$, a multifunctional tissue repair and regeneration peptide

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Studies in various animal models of disease and repair with thymosin $\beta_4$ (T$\beta_4$), the major actin-sequestering molecule in mammalian cells, have provided the scientific foundation for the ongoing dermal, corneal, and cardiac wound repair multicenter clinical trials. T$\beta_4$ has of multiple biological activities, which include down-regulation of inflammatory chemokines and cytokines, and promotion of cell migration, blood vessel formation, cell survival, and stem cell maturation. All of these activities contribute to the multiple wound healing properties that have been observed in animal studies. This paper reviews and discusses the topical and systemic uses of T$\beta_4$ in various animal models that demonstrate its potential for clinical use.

Keywords: thymosin $\beta_4$; wound repair; tissue regeneration; inflammation; apoptosis; re-epithelialization

Introduction

Thymosin $\beta_4$ (T$\beta_4$) is a small, naturally occurring peptide found in almost all cells with relatively higher levels in circulating cells, such as platelets and white cells.\textsuperscript{1} It is highly significant that T$\beta_4$ is in platelets because these are the first cells to arrive at a site of injury where these cells release various factors that initiate the repair process. T$\beta_4$ levels are high in wound fluid, confirming that it is naturally present at wound sites and could function to promote dermal repair (Table 1). Although many of the factors released by the platelets are important in cell growth, T$\beta_4$ is not a growth factor, i.e., it does not promote cell growth. In fact, T$\beta_4$ is even smaller than standard growth factors which are generally similar in size to each other (4964 Da vs. 14,000 to 16,000 Da, respectively). Also, unlike growth factors, it does not bind to heparin which is ubiquitously present in tissues; therefore, T$\beta_4$ can freely diffuse deeply into tissues to promote angiogenesis, cell migration, re-epithelialization, and down-regulate inflammation, among other effects.\textsuperscript{1–17} Furthermore, T$\beta_4$ is present inside all cells and is not secreted, while growth factors are secreted, stored in the extracellular matrices outside of the cells, and only produced by certain cells. Finally, there are many growth factors and variants. Growth factors act in a cell-type-specific manner in their interactions and activity, and mainly only promote cell growth and migration, while T$\beta_4$ acts on many cell types and has many different biological effects beyond growth and migration, including being anti-apoptotic, antimicrobial, and antifibrotic (Table 1).\textsuperscript{18–22} These differences distinguish T$\beta_4$ both structurally and functionally from the families of growth factors and suggest that it may have a more beneficial role in wound repair.

There are many properties of T$\beta_4$ that are important in wound repair. As a multifunctional protein, T$\beta_4$ is important at multiple stages of wound repair in a variety of tissues. Table 1 provides a list of known activities of T$\beta_4$ related to tissue repair and regeneration. Platelet-released T$\beta_4$ has antimicrobial properties which help to fight/prevent infection in wound sites.\textsuperscript{21} An additional activity of T$\beta_4$ at the early wound site is its ability to promote cell migration, including re-epithelialization, which has been demonstrated for ocular and dermal wounds.\textsuperscript{3–8} While T$\beta_4$ promotes endothelial and epithelial cell migration, it decreases infiltration of white blood cells (decreases inflammation) and thus
Table 1. Key thymosin β₄ activities important in wound repair

| Activity                          | Significance/importance                                                                 | References |
|----------------------------------|----------------------------------------------------------------------------------------|------------|
| Actin binding                    | Cell shape and behavior, reduces viscosity in cystic fibrosis sputum                    | 1–3        |
| Migration                        | Wound healing (re-epithelialization), organ formation, blood vessel formation           | 3–8        |
| Blood vessel formation (angiogenesis) | Wound healing, organ formation, nerve growth, tissue survival                          | 9–13       |
| Anti-inflammation                | Reduces swelling, pain, tissue damage, death (due to septic shock)                     | 14–17      |
| Anti-apoptosis/survival          | Prevents cell death from infections, toxins, or loss of blood supply (protects heart and nerves after loss of blood supply) | 17–20      |
| Antimicrobial                    | Prevents bacterial infection and tissue damage                                          | 21         |
| Wound healing                    | Dermal, corneal, cardiac                                                               | 1,10,11,15,20,24 |
| Antifibrotic                     | Prevents scarring and tissue damage                                                    | 22         |
| Stem cell differentiation        | Wound healing, hair growth, cardiac repair, promotes stem cell maturation               | 1,13,23    |

prevents swelling and further tissue damage. In nerve cells, in the eye, and in the heart after myocardial infarction (which is essentially a wound to the heart), Tβ₄ reduces cell death; thus, it promotes cell survival. Scar formation is reduced in the presence of Tβ₄ which helps to maintain tissue function, especially in cardiac repair after myocardial infarction. Tβ₄ promotes stem cell differentiation and this is also important in the repair of the heart and in the skin where blood vessels, skin, and hair follicles are continuously replaced. Another major activity of Tβ₄ important in dermal wound healing and in recovery from myocardial infarction is the ability to promote new blood vessel formation (angiogenesis). New blood vessels are needed to supply oxygen and nutrients to the tissues for maintenance, to promote growth, and to remove waste products. Interestingly, new blood vessels have not been reported in wounds in the eye that have been treated with Tβ₄, which is important because the eye stroma is vascular and new vessels would potentially damage or block vision. In summary, all of the actions of Tβ₄ are ideal for promotion of wound healing in various types of tissues.

**Topical use of Tβ₄ in animal models**

Tβ₄ has been used as a topical treatment both on the skin and in the eyes (Table 2). In the eye, both alkali injury and heptanol debridement have been successfully treated in mice and rats, respectively. Tβ₄ is applied to the eye as drops of liquid (5 µg/5 µL) twice per day. The corneal epithelium rapidly migrates over the surface of the cornea to replace the destroyed or removed tissue. With the alkali (burn) injury, there is considerable inflammation, which is also alleviated by topical Tβ₄. Tβ₄ appears to act in reducing inflammation by reducing matrix metalloproteinases (MMPs), blocking TNF alpha-stimulated cytokine release, and activating nF kappa b. Tβ₄ also increases the production of laminin-5 which promotes cell migration and cell–cell contacts in the eye. Decreased apoptosis is also observed in eye wounds treated with Tβ₄. Although Tβ₄ has been shown to promote angiogenesis in angiogenesis assays and in dermal wounds, no angiogenesis is observed in the eye as mentioned earlier even when the eyes are treated every day for 30 days.

Full thickness punch dermal wounds have been used in rats and in mice to study the wound healing activity of Tβ₄ in both normal and healing-impaired models (Table 2). Tβ₄ is applied either in PBS or in a hydrogel in doses ranging from 5 to 50 µg/50 µL/wound. Generally because of the formation of the crust, Tβ₄ is applied at the time of wounding (day 0 and after 48 h only), but in...
one case where bandages were used it was applied every day. In dermal wound healing, Tβ₄ increases keratinocyte migration, accelerates collagen deposition, and increases angiogenesis. Tβ₄ accelerates wound healing in impaired models of healing, such as diabetic and aged mice and steroid-treated rats. Both synthetic and recombinant Tβ₄ work well in dermal wounds and one Tβ₄-derived peptide containing the actin-binding site also promotes murine dermal repair.11,12 This same site also promotes cell migration, adhesion, and angiogenesis.26

An unexpected finding with the dermal wound studies was an increase in hair growth around the wound area treated with Tβ₄.23 The role of Tβ₄ in hair growth was confirmed by additional studies. When Tβ₄ is applied topically every other day to shaved rats or shaved or depilated mice at 2.5–5.0 μg/50 μL of hydrogel, hair growth is accelerated. The hair is generally thicker and darker in color than the surrounding normal hair. Nude mice also respond with increased hair growth. Based on in vitro studies using isolated hair follicle stem cells and hair follicle rudiments, hair growth appears to be increased due to increased migration and subsequent differentiation of stem cells from the bulge region. There appears to be an activation of existing follicles rather than generation of new follicles.

**Systemic use in animal models**

Tβ₄ has been used systemically in various models of cardiovascular disease, neural damage, dermal injury, and septic shock with doses ranging from 400 ng/animal (for intracardiac injection) to 15 mg/animal (for retroinfusion in 25 kg German pigs) (Table 3).10,17,19,20,27 Tβ₄ is cardioprotective. It promotes cardiac repair in a murine coronary ligation model and prevents vascular damage in an ovine reperfusion injury model.20,27 In the heart, it functions in part by activating integrin-linked kinase (ILK and Akt activity) and by promoting cardiac cell migration, survival, and stem cell activation, and differentiation resulting in decreased fibrosis, improved myocardial function, and enhanced survival.13,20,27,28 When Tβ₄ is retroinfused into the anterior interventricular vein in a hypoxia-reoxygenation pig model, it decreases infarct size and inflammatory cell influx.27 The effects with exogenously administered Tβ₄ mimicked those observed with embryonic endothelial progenitor cell administration in preventing tissue damage. The authors suggest that a single regional administration of Tβ₄ can protect cardiac tissue from reperfusion injury and that the short-term cardioprotection obtained from embryonic endothelial progenitor cells

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**Table 2. Studies with topical Tβ₄**

| Model                          | Dosage               | Frequency        | Outcome                                                                 | Reference |
|-------------------------------|----------------------|------------------|-------------------------------------------------------------------------|-----------|
| Rat cornea heptanol debridement | 5 μg/5 μL            | Twice per day    | Corneal repair                                                           | 24        |
| Mouse cornea-alkali injury     | 5 μg/5 μL            | Twice per day    | Decreased inflammation, decreased chemokines, and cytokines accelerated epithelialization | 15        |
| Rat dermal 8 mm punch wounds   | 5 μg/50 μL PBS 25 μL/50 μL hydrogel | Every day   | Increased wound closure, accelerated collagen deposition, angiogenesis | 10        |
| Mouse dermal Db/db, aged 3 mm punch wounds | 5 μg/50 μL PBS 25 μL/50 μL hydrogel | 0, 48 h | Increased wound closure at 7 days                                        | 11        |
| Mice dermal 3 mm punch wounds  | Recombinant 5 μg/50 μL hydrogel | 0, 48 h | Increased wound closure at 7 days                                        | 12        |
| Mice-shaved or depilated       | 2.5 μg/50 μL hydrogel | Every other day  | Increased hair regrowth                                                  | 23        |
| Rat-shaved                     | 5 μg/50 μL hydrogel  | Every other day  | Increased hair regrowth                                                  | 23        |
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Table 3. Studies with systemic Tβ₄

| Model                              | Tβ₄ dosage                  | Frequency       | Route of administration                                      | Outcome                                      | Reference |
|------------------------------------|-----------------------------|-----------------|-------------------------------------------------------------|----------------------------------------------|-----------|
| Pigs-reperfusion model             | 15 mg/25 kg animal          | One time        | Retroinfusion into anterior interventricular vein            | Cardiac protection                          | 27        |
|                                    |                             |                 |                                                             |                                              |           |
| Mice-permanent ligation model      | Intracardiac 400 ng/animal or intraperitoneal 150 μg/animal | Every 3 days    | Systemic, intracardiac or both systemic + intracardiac injection | Enhanced myocyte survival + improved cardiac function | 20        |
| Rats with kainic acid via cannula in lateral cerebral ventricle | 10 μM (in 10 μL) = 4.3 μg/animal | Twice per day for 5 days | Via cannula in brain                                        | Prevented toxic effect of kainic acid         | 19        |
| Rats-LPS LD50 endotoxin model      | 100 μg/ animal              | At 0, 2 and 4 h after LD50 LPS | IP injection                                                | Reduced mortality, decrease blood levels of inflammatory cytokines | 17        |
| Rat dermal wound 8 mm punch        | 60 μg/rat                   | At 0, 2 days    | IP injection                                                | Improved wound healing                       | 10        |
| Mice with experimental autoimmune encephalomyelitis | 6 mg/kg animal             | Every 3 days for 5 treatments | IP injection                                                | Neurological functional recovery              | 29        |

Various toxicity studies show no toxic effects with doses as high as 60 mg/kg for dogs and rats administered IV, at 50 mg/kg for rats and monkeys administered IV, and for 250 mg/kg for rats and monkeys administered IV. All doses were well tolerated in all species.³²

administered in reperfusion injury is due to Tβ₄.²⁸ Tβ₄ had already been shown to increase cardiac stem cell migration and differentiation in vivo, which may explain part of the mechanism of how Tβ₄ is cardioprotective.¹³

Consistent with the anti-inflammatory activity of Tβ₄ and the role of proinflammatory cytokines in septic shock, Tβ₄ administered immediately following endotoxin and at 2 and at 4 h after a dose of endotoxin reduced lethality and down-regulated inflammatory mediators in endotoxin-induced septic shock in rats.¹⁷ Interestingly, with the administration of LPS to the rats to induce septic shock, there is a decrease in circulating levels of Tβ₄. In patients given low doses of endotoxin and in patients with septic shock, there is a similar reduction in circulating levels of Tβ₄. This suggests that Tβ₄ may be part of the host response to counteract sepsis and that it may have use as a therapeutic in the treatment of sepsis.

Tβ₄ is also neuroprotective both in vivo and in vitro. Intracerebroventricular administration of Tβ₄ prevented the loss of hippocampal neurons after kainic acid treatment. Tβ₄ (10 μL of a 10 μM solution) was administered twice a day for 5 days after the kainic acid injection.¹⁹ It can also
promote functional recovery in mice with experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis. In this model, there was less inflammation and an increase in mature oligodendrocytes after the mice received 6 mg/kg intraperitoneally 24 h after infection and every 3 days for four more additional treatments. In vitro, Tβ4 also protected cortical neurons and rat hippocampal slices from glutamate-induced toxicity and cerebral cortex astrocytes from ethanol toxicity. Interestingly, Tβ4 is induced in the brain following ischemia and may be a naturally occurring repair factor in the brain.

Conclusion

These studies demonstrate that in experimental models of disease and repair, Tβ4 is able to prevent cell loss and promote repair, suggesting its potential as a therapeutic for various types of injuries. Tβ4 acts by promoting cell migration, reducing inflammation, promoting stem cell differentiation, reducing apoptosis, and promoting angiogenesis. The observed reduction in fibrosis is particularly important for cardiac and neural repair. It should be noted that various nonclinical pharmacology and toxicology studies in dogs, rats, and monkeys have demonstrated that systemic administration of Tβ4 is safe and well-tolerated by the animals.

Conflicts of interest

HK is a consultant to RegeneRx Biopharmaceuticals Inc., which is currently conducting clinical trials on Tβ4 for various indications.

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