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Animal studies with thymosin β_4, a multifunctional tissue repair and regeneration peptide

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Studies in various animal models of disease and repair with thymosin β_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 β_4; wound repair; tissue regeneration; inflammation; apoptosis; re-epithelialization

Introduction

Thymosin β_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.[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.[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).[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.[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.[3–8] While $T\beta_4$ promotes endothelial and epithelial cell migration, it decreases infiltration of white blood cells (decreases inflammation) and thus

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Table 1. Key thymosin β_4 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.[14–17] In nerve cells, in the eye, and in the heart after myocardial infarction (which is essentially a wound to the heart), $T\beta_4$ reduces cell death; thus, it promotes cell survival.[17–20] Scar formation is reduced in the presence of $T\beta_4$ which helps to maintain tissue function, especially in cardiac repair after myocardial infarction. $T\beta_4$ 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.[1,13,23] Another major activity of $T\beta_4$ important in dermal wound healing and in recovery from myocardial infarction is the ability to promote new blood vessel formation (angiogenesis).[9–13] 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 $T\beta_4$, 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 $T\beta_4$ are ideal for promotion of wound healing in various types of tissues.

Topical use of $T\beta_4$ in animal models

$T\beta_4$ has been used as a topical treatment both on the skin and in the eyes (Table 2).[15,24] In the eye, both alkali injury and heptanol debridement have been successfully treated in mice and rats, respectively. $T\beta_4$ 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 $T\beta_4$.[15] $T\beta_4$ appears to act in reducing inflammation by reducing matrix metalloproteinases (MMPs), blocking TNF alpha-stimulated cytokine release, and activating nF kappa b.[15,16] $T\beta_4$ also increases the production of laminin-5 which promotes cell migration and cell–cell contacts in the eye.[25] Decreased apoptosis is also observed in eye wounds treated with $T\beta_4$.[18] Although $T\beta_4$ has been shown to promote angiogenesis in angiogenesis assays and in dermal wounds,[9–13] 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 $T\beta_4$ in both normal and healing-impaired models (Table 2).[10–12] $T\beta_4$ 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, $T\beta_4$ is applied at the time of wounding (day 0 and after 48 h only), but in

Table 2. Studies with topical $T\beta_4$

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 PBS	0, 48 h	Increased wound closure at 7 days	12
Mice-shaved or dipliated	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

one case where bandages were used it was applied every day. In dermal wound healing, $T\beta_4$ increases keratinocyte migration, accelerates collagen deposition, and increases angiogenesis. $T\beta_4$ accelerates wound healing in impaired models of healing, such as diabetic and aged mice and steroid-treated rats. Both synthetic and recombinant $T\beta_4$ work well in dermal wounds and one $T\beta_4$-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 $T\beta_4$.[23] The role of $T\beta_4$ in hair growth was confirmed by additional studies. When $T\beta_4$ 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

$T\beta_4$ 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] $T\beta_4$ 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 $T\beta_4$ 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 $T\beta_4$ mimicked those observed with embryonic endothelial progenitor cell administration in preventing tissue damage. The authors suggest that a single regional administration of $T\beta_4$ can protect cardiac tissue from reperfusion injury and that the short-term cardioprotection obtained from embryonic endothelial progenitor cells

Table 3. Studies with systemic $T\beta_4$

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 wth 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 $T\beta_4$.[28] $T\beta_4$ had already been shown to increase cardiac stem cell migration and differentiation *in vivo*, which may explain part of the mechanism of how $T\beta_4$ is cardioprotective.[13]

Consistent with the anti-inflammatory activity of $T\beta_4$ and the role of proinflammatory cytokines in septic shock, $T\beta_4$ 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 $T\beta_4$. In patients given low doses of endotoxin and in patients with septic shock, there is a similar reduction in circulating levels of $T\beta_4$. This suggests that $T\beta_4$ may be part of the host response to counteract sepsis and that it may have use as a therapeutic in the treatment of sepsis.

$T\beta_4$ is also neuroprotective both *in vivo* and *in vitro*. Intracerebroventricular administration of $T\beta_4$ prevented the loss of hippocampal neurons after kainic acid treatment. $T\beta_4$ (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.[29] 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*, $T\beta_4$ also protected cortical neurons and rat hippocampal slices from glutamate-induced toxicity and cerebral cortex astrocytes from ethanol toxicity.[19,30] Interestingly, $T\beta_4$ is induced in the brain following ischemia and may be a naturally occurring repair factor in the brain.[31]

Conclusion

These studies demonstrate that in experimental models of disease and repair, $T\beta_4$ is able to prevent cell loss and promote repair, suggesting its potential as a therapeutic for various types of injuries. $T\beta_4$ 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 $T\beta_4$ is safe and well-tolerated by the animals.[32]

Conflicts of interest

HK is a consultant to RegeneRx Bipharmaceuticals Inc., which is currently conduting clinical trials on $T\beta_4$ for various indications.

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