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Thymosin β4: structure, function, and biological properties supporting current and future clinical applications

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Published studies have described a number of physiological properties and cellular functions of thymosin β4 (Tβ4), the major G-actin-sequestering molecule in mammalian cells. Those activities include the promotion of cell migration, blood vessel formation, cell survival, stem cell differentiation, the modulation of cytokines, chemokines, and specific proteases, the upregulation of matrix molecules and gene expression, and the downregulation of a major nuclear transcription factor. Such properties have provided the scientific rationale for a number of ongoing and planned dermal, corneal, cardiac clinical trials evaluating the tissue protective, regenerative and repair potential of Tβ4, and direction for future clinical applications in the treatment of diseases of the central nervous system, lung inflammatory disease, and sepsis. A special emphasis is placed on the development of Tβ4 in the treatment of patients with ST elevation myocardial infarction in combination with percutaneous coronary intervention.

Keywords: thymosin β4; tissue protection; tissue repair; tissue regeneration; apoptosis; inflammation; cell survival

Introduction

The beta-thymosins (β-thymosins) comprise a family of structurally related, highly conserved amino-acid sequences in species ranging from mammals to echinoderms.[1] Of the 16 known family members, thymosin β4 (Tβ4), thymosin β10 (Tβ10), and thymosin β15 (Tβ15) are found in man. Although highly homologous in structure (43–44 amino-acid residues), these three acidic polypeptides are derived from different gene products and thus are biochemically and functionally distinct molecules.[2,3] Tβ4 is a small 43-amino acid intracellular peptide that was first isolated from bovine thymus tissue. Its N-terminal sequence, represented by *N*-acetyl-seryl-aspartyl-lysyl-proline (ac-SDKP), can be generated by a single cleavage step of the Pro-Asp bond, employing either prolyloligopeptidase or an AspN-like protease. An oxidizable methionine residue is located at position 6, and the glutaminyl donors in transglutaminase reactions are located at amino-acid positions 23, 36, and 39.[3,4]

Tβ4 has a dynamic, unstructured, and flexible conformation. NMR studies have shown that Tβ4 is mostly unstructured in aqueous solution with some preferential α helical conformations observed.[5,6] In a collaboration with a major university in the United States, it was shown by circular dichromism that Tβ4 in aqueous solution and at pH and temperature ranges between 4.5–7.5 and 5–40° C, respectively, displays no well-defined secondary structure. However, the initial interaction with G-actin by way of the ^{17}LKKTET^{22} sequence induces the folding of Tβ4 and the formation of structured N- and C-terminal helices.[3,7] This interaction with G-actin, forming a 1:1 complex, is the process by which Tβ4 by sequestration maintains a large pool of actin monomers, controlling the assembly and disassembly of actin filaments that regulate the dynamics of the actin cytoskeleton. Thus, the mechanism by which Tβ4 influences cell differentiation, morphogenesis, migration, and organogenesis is believed to be linked to its maintaining a dynamic equilibrium between G- and F-actin, essential for the rapid reorganization of the cytoskeleton.

Tβ4 is the most abundant member in most cell types, present in concentrations as high as 0.3–0.4 mM in a number of formed elements including

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cell fragments (e.g., blood platelets), cell types (e.g., macrophages and white blood cells), and tissues (e.g., thymus and spleen), and representing approximately 70–80% of the total thymosin content.[3,8,9] Given its high concentration and ubiquitous distribution, it is not surprising that Tβ4 functions as an important intracellular structural element of the cytoskeleton and, when released from cells by either secretion, cell lysis, or necrosis, serves as a moonlighting protein to repair and regenerate damaged tissue.[3,10] The intracellular and extracellular properties of this major actin-sequestering peptide contribute to the many biological activities that support the use of Tβ4 in the clinic.

Biological activities providing scientific rationale for use in man

Nonclinical studies have shown several key biological activities for Tβ4 to promote wound repair, tissue protection, and regeneration in the skin, eye, heart, and central nervous system.

Tβ4 promotes cell migration and adhesion dynamics

Tβ4 is the major actin-sequestering molecule in all eukaryotic cells and a potent regulator of actin polymerization in mammals. Actin participates in a number of cellular functions including not only cell motility but also cell division, cytokinesis, vesicle and organelle movement, microtubule organization, and the establishment and maintenance of cell junctions and shape. Tβ4 binds to G-actin in an extended conformation, blocking by steric hindrance, salt-induced actin polymerization. The regulation of polymerization and depolymerization of actin subunits is a key mechanism by which Tβ4 enables cells to migrate.[3,10–13] Cell migration is also influenced at the gene level by upregulation of the expression of laminin-5 by Tβ4.[14] Laminin-5 is not only a component of anchoring filaments, localized in the basement membrane region of the skin, cornea, conjunctiva, and other tissues, but also a unique subepithelial basement membrane protein believed to be one of the best ligands for keratinocyte adhesion and migration.[14–16] Another indirect mechanism of cell migration, not related to its direct effects on the actin cytoskeleton, is Tβ4's ability to activate Akt by forming a functional complex with PINCH and ILK. Akt plays a critical role in promoting not only cell growth and survival but also cell motility.[17]

Tβ4 promotes angiogenesis and cell differentiation

Tβ4 is a potent angiogenic molecule and has been shown to stimulate angiogenesis by differentiation and directional migration of endothelial cells and tube formation.[13,18–21] The motif for this activity was determined to be the central actin-binding domain, ^{17}LKKTETQ^{23} of Tβ4.[21,22] Yet, Tβ4 is not alone in this regard. Endoproteinase cleavage of the N-terminal sequence of Tβ4 leads to the release of the antifibrotic and proangiogenic tetrapeptide, Ac-SDKP, which stimulates endothelial migration and differentiation, indicating that there is duplication of the angiogenic properties shared between Ac-SDKP and its known sole precursor, Tβ4.[3]

In a cardiac-specific Tβ4 knock-down mouse model, Tβ4 was shown to promote coronary vessel development and collateral growth not only during embryonic development but also from the adult epicardium by stimulating epicardial vascular progenitors, which migrate and differentiate into smooth muscle and endothelial cells.[23]

Also Tβ4 has been shown in various rodent models to promote stem cell migration and differentiation into keratinocytes and hair follicles in the bulge region, inducing dermal repair and increased hair growth, respectively.[24,25]

Tβ4 prevents apoptosis, promotes cell survival, and tissue regeneration

Tβ4 has been shown to diminish the proapoptotic effect of ethanol on human corneal epithelial cells *in vitro* by decreasing damaging mitochondrial alterations and cytochrome *c* release from mitochondria, increasing bcl-2 expression and decreasing caspase activation.[26]

Tβ4 reduces apoptosis and induces survival genes. For example, it has been shown to inhibit endothelial apoptosis and activate the phosphoinositide/Akt cell survival signaling pathway in cardiomyocytes.[28] It supports cardiac regeneration by inhibiting myocardial and endothelial cell death after infarction, inducing vessel growth and myocardial progenitor mobilization. Remarkably, Tβ4 is the only known molecule to initiate organ wide activation of the embryonic coronary development program in adult mammalian hearts.[17,28,29]

Table 1. Nonclinical toxicology and safety pharmacology studies[a]

Acute Intravenous (IV) Tox Study in Rats	28-Day Tox and TK Study in Beagle Dogs	26-Week Dermal Tox and TK Study in the Rabbit Abraded Skin Model
Maximum Dose Tox Study in Rats	10-Day Tox Study in Beagle Dogs	Ames Mutation Assay
Acute IV Tox and Pharmacokinetic (PK) Study in Monkeys (cynomolgus)	Acute Oral Tox and PK Study in Rats	Mouse Lymphoma Mutagenesis Assay
Maximum Dose Tox Study in Monkeys	Acute Inhalation Tox Study in Rats	Mouse Micronucleus Genetic Mutation Assay
7-Day Subcutaneous (SC) Tox Study in Rats	Increasing Dose Inhalation Tox Study in Monkeys	28-Day Topical Ocular Toxicity Study in New Zealand White Rabbits
28-Day SC Tox Study in Rats	28-Day Dermal Tox and Efficacy Study in the Rabbit Abraded Skin Model	Cardiovascular and Respiratory IV Safety Pharmacology Study in Beagle Dogs
28-Day IV Tox and Toxicokinetic (TK) Study in Rats	13-Week Dermal Tox and TK Study in the Rat Abraded Skin Model	Dermal Sensitization Potential in Guinea Pigs
In Vitro Evaluation of Hemolytic (blood) and Flocculation (plasma protein) Potential	Acute IV Tox and PK Study in Rats	Reserved

[a]All studies performed in accordance with Good Laboratory Practice regulations (21 CFR Part 58) of the FDA.

Tβ4 reduces inflammation and inhibits influx and adhesion of inflammatory cells

In models of dermal, corneal eye and cardiac injury and endotoxin-induced sepsis, Tβ4 has been shown to downregulate inflammatory mediators and reduce inflammatory cell infiltrates.[28,30–34] These activities have been linked to its ability to decrease matrix metalloproteinases (MMPs), gene transcript levels of a number of proinflammatory cytokines and chemokines, and suppress the activation and translocation of the nuclear transcription factor, NFκB.[35] Its effect on inflammation-reduction can also be attributed to the fact that Tβ4 upregulates IL-10, which has immune-response downregulatory properties that include the suppression of proinflammatory cytokines and MHC class II expression on monocytes.

Nonclinical toxicology studies providing evidence of safety for use in man

Based on FDA recommendations and various guidance documents developed by the International Conference of Harmonization (ICH), 23 nonclinical studies, identified in Table 1, have been performed to date that demonstrate the safety of Tβ4 for its current and planned uses in man. Additional nonclinical safety studies will be performed to assess effects of long-term repeat dosing, absorption, distribution, metabolism, and excretion (ADME), reproduction toxicity, and safety pharmacology for assessment of vital functions related to the cardiovascular, respiratory, and central nervous systems. In addition, a 2-year carcinogenicity study to assess the tumorigenic potential of Tβ4 may be performed as well. Although there has been no previous demonstration of carcinogenic potential, no structure–activity relationship suggesting carcinogenic risk, no evidence of preneoplastic lesions in repeated dose toxicity studies, and no evidence of prolonged retention with Tβ4, most pharmaceuticals, and particularly those that are administered chronically, are tested for their carcinogenic potential before widespread use in man.

Clinical studies—dermal wound healing and repair

Tβ4's dermal wound healing and repair properties are evidenced in the rat full-thickness excisional rat model and impaired-healing models (db/db diabetic, steroid-immunosuppressed, and aged mice) by the promotion of keratinocyte and endothelial cell migration, rapid collagen deposition,

stimulation of angiogenesis, upregulation of laminin-5, and suppression of proinflammatory mediators.[14,30,36] Based on those properties, Tβ4, formulated as topical dermal gel (RGN-137), has the potential to be studied in patients with chronic cutaneous ulcers and other types of dermal wounds, such as those observed in patients with epidermolysis bullosa (EB).[37] Chronic cutaneous ulcers are wounds that have failed to proceed through an orderly and timely orchestrated series of events to produce a durable structural, functional, and cosmetic closure despite accepted standard care regimens.[38] Because they differ in their pathophysiology and the way in which they respond to treatment, separate trials are warranted for each type of chronic ulcer to be studied. The outcomes of the two studies in patients with pressure and venous stasis ulcers will be described. A Phase 2 controlled clinical study in patients with two of the more severe subtypes of EB, dystrophic and junctional, is ongoing and will not be presented here.

Pressure ulcers

Pressure ulcers are localized areas of tissue necrosis that occur when soft tissue is compressed between a bony prominence and an external surface for a prolonged period of time. The standard of care for these patients involves the off-loading of pressure, debridement of necrotic tissue, wound cleansing, adequate oral and/or parenteral nutritional support and fluid intake, application of appropriate wound dressings, and proper bowel and bladder hygiene to reduce risk of fecal and urinary contamination of the wound. Healing of pressure ulcers is a lengthy process that requires a large amount of health care resources, and the recurrence rate is high.

The pressure ulcer clinical trial was a Phase 2, multicenter, randomized, double-blind, placebo-controlled, dose–response study evaluating the safety tolerability and wound healing effectiveness of Tβ4 in three escalating concentrations when applied once daily for up to 84 days. Dose escalation occurred sequentially by cohort and was preceded by a blinded review of safety after all subjects in a cohort had completed at least 28 days of treatment and safety evaluations. The study population consisted of patients between 19 and 84 years of age (inclusive) with at least 1 pressure ulcer, classified as Stage III or IV per the International Association of Enterostomal Therapy (IAET), present and stable for at least 1 month prior to enrollment and with a wound area, measured by digital planimetry, of 5–70 cm^2.[39] Seventy-one patients were randomized to receive the study drug as follows: 18 patients received placebo, 17 patients received the low dose (0.01% Tβ4), 18 patients received the mid dose (0.02% Tβ4), and 18 patients received the high dose (0.1% Tβ4). Overall, Tβ4 was well tolerated. No serious adverse events were deemed related to the study drug. No dose-dependent relationship was noted for either the incidence or the severity of adverse events, nor noted for any other safety parameter (application site abnormalities, clinical chemistry, hematology, urinalysis, physical exam, and vital signs). On the efficacy measures, all of the Tβ4 doses performed similarly to placebo. Patients treated with the mid dose, however, showed a 17% rate of wound healing, which was the highest among the active doses. Improved ulcer healing in patients receiving the mid dose, observed in the first 9 weeks of treatment, was equal to the placebo by the end of treatment.

Venous stasis ulcers

Venous stasis ulcers develop on the ankle or lower leg in patients with chronic vascular disease and may be caused by an abnormal calf muscle pump due to incompetent veins or valves of the lower extremities, muscular dysfunction, limited mobility, or any combination of the three. In these patients, blood flow to the lower extremities is impaired, leading to edema, mild redness, and scaling of the skin that gradually progresses to ulceration.[40,41] Despite standard therapy with compression dressings, only 50–60% of venous ulcers will heal in 6 months of treatment.[42,43]

The venous stasis ulcer Phase 2 trial was designed similarly to the pressure ulcer trial. Key inclusion criteria include patients between 18 and 79 years of age, presenting with evidence of venous insufficiency as documented by venous duplex scanning or impedance plethysmography. Each patient enrolled was required to have a stable venous leg ulceration with a surface area between 3 and 30 cm^2. Standard of care included the use of a dressing with a compression pressure at least 30 mm Hg at the ankle and decreasing in pressure up to below the knee. In this study, a total of 72 patients from eight centers in Italy and Poland were randomized to receive study medication: 17 patients received placebo, 19 patients received Tβ4 0.01%, 18 patients received

Table 2. A randomized, double blind, placebo-controlled study of the efficacy and safety of Tβ4 in the treatment of patients' venous stasis ulcers (VSU)

Design (preliminary)
• VSU patients with mild to moderate disease (CEAP classification)
• Target ulcer surface area between 2 and 20 cm^2
• Treatment to concentrate on initial stage of healing for up 56 days
• One hundred and forty ($N = 140$) eligible patients would enroll sequentially and be randomized to receive either Tβ4 0.03% (w/w) or placebo in a 1:1 ratio.
Proposed endpoints (preliminary)
• 25% improvement in healing incidence in Tβ4 patients versus placebo
• 40% improvement in time-to-healing
• Improvement in Q of L measures (instrument TBD)
• Safety profile to be established for the dose received

Tβ4 0.03%, and 18 patients received Tβ4 0.1%. The safety profile of all doses of Tβ4 was acceptable, and the number of patients experiencing adverse events (AEs) was similar across all treatment groups. The majority of AEs were mild and not related to study medication. All efficacy parameters were secondary study objectives and considered exploratory variables with no confirmatory statistical analysis done. The two key parameters evaluated, incidence of healing and time to healing, indicated more patients achieved healing in the Tβ4 0.03% group (six patients [33.3%] compared to four patients [23.5%] in the placebo group, three patients [15.8%] in the Tβ4 0.01% group, three patients [16.7%] in the Tβ4 0.1% group). Median time to healing could not be calculated due to the small number of patients with healed ulcers. In this study, the incidence of healing was shown to decrease with increased disease severity and baseline ulcer area, which was also associated with a longer time to healing. In the full analysis population, the mid dose showed both an increased incidence of healing and a faster time to healing compared to placebo. Taking this into consideration and those factors affecting healing outcomes, a Phase 3a clinical trial in venous stasis ulcers, described in Table 2, is being considered.

Clinical studies—corneal wound healing and repair

Corneal wound repair is linked to migration of corneal epithelial cells, tight cell–cell junctions, epithelial adhesion, and decreased inflammatory response, which must be controlled to ensure proper healing and the best visual outcome. Tβ4 accelerates epithelial cell migration and re-epithelialization after alkaline and alcohol injuries and scrape wounding in a dose-dependent manner in rat corneal wound models.[31,32] Tβ4 markedly reduces the inflammatory response and edema seen in the corneal stroma of rats after alkali injury. In these wound models, Tβ4 was shown to reduce a number of proinflammatory cytokines and chemokines, suppress the activation of NFκB, and modulate the production of matrix metalloproteinases, MMP-1, -2, and -9.[31–33,35] Tβ4 has also been shown to inhibit corneal epithelial cell apoptosis.[26] TEM of corneas treated with Tβ4 following heptanol injury showed a more regular alignment of epithelial intercellular junctions and less vacuolization.[33] These findings suggest that Tβ4 may have the ability to restore the normal cytoarchitectural structure following treatment in man. With a strong scientific rationale to evaluate the wound healing and repair properties of Tβ4 in human clinical trials, Tβ4 was formulated as a sterile, preservative-free eye drop (RGN-259), suitable for topical application in man.

Compassionate uses

1. A female vitrectomy patient with diabetes, heart and liver disease was treated with Tβ4 approximately 3 weeks post vitrectomy for her nonhealing corneal epithelial wound. The wound appeared to completely heal, as evidenced by slit lamp examination (SLE) on the 11th day of a 14-day treatment period; however, the epithelial tissue sloughed posttreatment without a known cause.
2. A vitrectomy patient with a nonhealing corneal epithelial wound was treated with Tβ4. Although the patient's cornea did not completely heal during a 14-day treatment period, the epithelium subjectively improved in quality.
3. Four patients with nonhealing, long-standing neurotrophic ulcers were treated with Tβ4 for 28 consecutive days. In all patients, the ulcers had either healed completely or demonstrated

significant improvement.[44] Up to 10 patients will be enrolled in this compassionate study.

Phase 2 clinical trial in vitrectomy patients

The safety and efficacy of Tβ4 was evaluated in a randomized, double-mask, placebo-controlled, dose–response Phase 2 study. Tβ4 was used to treat diabetic patients' corneal wounds resulting from epithelial debridement during vitrectomy. A total of 36 patients were originally planned for three treatment groups of 12 patients each. Of the 25 patients enrolled in the first treatment group, 12 patients underwent corneal epithelial debridement for improved intraoperative fundus visualization and were randomly assigned to study drug with nine patients receiving treatment with Tβ4 0.01% (low dose) and three patients receiving placebo. Because of slow enrollment and the small number of subjects undergoing corneal epithelial debridement, the study was terminated before enrollment of the second treatment group. In general, the Tβ4 0.01% dose was safe and well tolerated with a safety profile comparable to placebo. The lower incidence of corneal edema and anterior chamber flare observed among patients in the Tβ4 treatment group compared with those receiving placebo is consistent with the known antiinflammatory properties of Tβ4. Evidence of wound healing as indicated by central corneal thickness was greater for patients treated with Tβ4 than for patients treated with placebo. Because of small sample size and data inconsistency, efficacy findings of time to wound healing between Tβ4 0.01% versus placebo treatments are inconclusive. Elevated baseline HbA_{1c} levels of greater than 9% among the majority of patients (six of nine) receiving Tβ4 may have also confounded efficacy results by possibly lengthening healing time compared with patients receiving placebo where none had baseline HbA_{1c} levels above 9%.

Other potential corneal wound healing and repair applications

Various nonmedical (the absence of an underlying pathology) and medical applications for use of Tβ4 in the cornea are shown in Table 3.

Table 3. Corneal wound healing applications

Nonmedical application
• Chemical burns
• Patients undergoing photorefractive keratectomy (PRK)
Medical applications
• Stage 2 patients (Mackie classification) with neurotrophic keratitis
• Patients with recurrent corneal erosions
• Patients with map-dot fingerprint and/or Fuch's corneal dystrophies
• Corneal transplants
• Patients undergoing phototherapeutic keratectomy (PTK) for anterior stromal corneal dystrophies

Clinical studies—cardiac, central nervous system, and pulmonary

Tβ4 has been formulated as an injectable solution to allow for its use in a number of clinical applications that would require systemic or local delivery. Its initial development has been described earlier.[45] Further refinement of this product, now referred to as RGN-352, has allowed it to be packaged as a refrigerator-stable, ready-to-inject, liquid formulation.

Phase 1 clinical trial in healthy volunteers

A randomized, double-blind, placebo-controlled single- and multiple-dose Phase 1 clinical trial, evaluating the safety and pharmacokinetics of the intravenous (iv) administration of Tβ4, was conducted. Dose groups (cohorts) of 10 subjects each were given a single dose of 42, 140, 420, and 1260 mg Tβ4 or placebo in a Tβ4-to-placebo ratio of 4:1. Following a blinded review of safety, subjects were given the same doses for 14 consecutive days and were evaluated during the course of treatment and at follow-up for incidence of treatment-emergent adverse events, clinical laboratory findings, vital signs, and ECG changes from baseline. Bloods were drawn for pharmacokinetic (PK) analysis and for an assessment of the potential of Tβ4 to elicit an antibody response. Results of those analyses are pending. Intravenous administration of Tβ4 appears to be safe and well-tolerated by all subjects with no dose limiting toxicity or serious adverse events reported. The most frequent adverse event (AE) observed in subjects following receipt of a single dose was an elevated CPK level, noted in both placebo- and Tβ4-treated subjects during outpatient visits, and which likely reflected the increased physical activity following confinement in the Phase 1 unit.

The most frequent AE reported in subjects receiving multiple doses was headache, noted in all dose groups including placebo. Increasing doses did not produce an increase in the incidence or severity of headache. None of the AEs were deemed probably or definitely related to the study drug. No clinically significant ECG findings were reported in subjects receiving Tβ4 or placebo. The proven safety of Tβ4 injectable solution sets the stage for its use in the clinical arena.

Cardiac indication—ST elevation myocardial infarction (STEMI)

Each year, in the United States alone, over 650,000 patients experience an acute, severe myocardial infarction typically due to complete occlusion of an epicardial coronary artery, detected on the ECG wave form as an ST-segment elevation. Survivors are often left with debilitating and permanent damage to the myocardium. Currently, there are no pharmacological agents on the market to prevent cardiac injury and promote repair after such a heart attack. Tβ4 has been shown to have a clear positive role in cardioprotection and repair. In the permanently ligated mouse and ischemia-reperfusion pig models, Tβ4 reduced damage after ischemic heart injury, protected posthypoxic cardiac tissue, decreased infarct size, reduced scar volume, decreased inflammation, promoted angiogenesis, improved ventricular function, and survival.[17,28,45] These studies demonstrate a strong rationale for use of Tβ4 to prevent cardiac damage, promote regeneration after an infarction, and maintain normal tissue function using a safe and well-tolerated agent, such as Tβ4.

A multicenter, double-blind, placebo-controlled, dose-escalation Phase 2 clinical trial is being considered to evaluate the safety, efficacy, and PK of Tβ4 when administered intravenously to patients receiving PCI (coronary angioplasty) for their STEMI. Four groups of approximately 170 eligible patients would each enroll nonsequentially. Each patient would have a baseline angiography, demonstrating a single lesion with a TIMI flow grade 0/1 in the infarct-related artery. Onset of symptoms to balloon time would not exceed 6 hours. Tβ4 would be administered by intravenous injection at the time of balloon deflation and possibly on two consecutive days thereafter. PK data, when available from the Phase 1 trial, will guide decisions regarding a dosing regimen in this Phase 2 trial. Safety criteria, including observation of AEs, would be assessed using NCI Common Toxicity Criteria v3. Efficacy evaluations would include MRI-derived infarct size by delayed Gd enhancement images, myocardial salvage and LVEF/LVF, and end systolic and diastolic volumes. Other assessments over a 6-month follow-up period would include laboratory biomarkers (CK-MB, Troponins, BNP), incidence of cardiac-related serious events, quality of life, and survival.

Central nervous system indications

Tβ4 is widely distributed in a majority or mammalian tissues and cell types, including those of the central nervous system. It is expressed in most neural cell types of the developing brain and in a subset of neurons and microglia.[46] Tβ4 is localized neuritis and regulates outgrowth of growing neuritis on neurons.[47,48] It is up-regulated in various pathological conditions such as focal ischemia, Alzheimer's disease, Huntington's disease, hippocampal denervation, and kainic acid induced seizure.[46] Its presence in the nervous system likely plays a role in neuroprotection, synaptogenesis, axon growth, cell migration, and plastic changes.[49]

Multiple sclerosis (MS)

In an experimental autoimmune encephalomyelitis animal model of MS, the systemic administration of Tβ4 improved neurological functional recovery. Along with this benefit, Tβ4 was shown to reduce inflammatory infiltrates and induce oligodendrogenesis, consistent with its antiinflammatory properties and ability to cause migration and differentiation of stem and progenitor cells.[50] Results of this study suggest that Tβ4 may be an important pharmacological therapy in the treatment of MS. An open-label, dose-titration study (Phase 1 or Phase 1/2) is under consideration that would broadly enroll primary progressive, secondary progressive, and relapse-remitting MS patients to assess the safety, clinical activity, and PK of Tβ4 injectable solution.

Ischemic stroke

Rats, who were subjected to middle cerebral artery occlusion (MCAo model), were treated by Tβ4 to assess improvements in neurologic function. The administration of Tβ4 24 h after stroke in the MCAo animal model improved functional outcome. A

significant increase in remyelinization was noted due to Tβ4 stimulating oligodendrocyte progenitor cells to migrate and differentiate into mature oligodendrocytes.[51] Future animal studies are warranted to assess, among other things, the timing of the administration of Tβ4 relative to stroke onset. Once obtained, a Phase 2 exploratory clinical trial in patients presenting with embolic (nonhemorrhagic) stroke would be warranted.

Spinal cord injury (SCI)

SCI is a devastating injury to millions of people worldwide, and there is no cure. The only pharmacologic intervention or treatment currently available is corticosteroid drugs, which reduces inflammation but also has numerous side effects and questioned efficacy.[52] Immediately after most spinal cord injuries, there is a relatively small region of the spinal cord where neurons and glial cells undergo necrotic cell death. Because muscles are innervated by multiple levels of the spinal cord, the resulting primary injury to overcome would seem to be minimal. However, a much greater region of spinal cord is subsequently damaged over time (secondary injury) due to the release of inflammatory cytokines and chemokines, free radicals, glutamate, and other small molecules and to apoptosis.[53–55] Treatment with Tβ4 should significantly reduce secondary injury due to its antiapoptotic, angiogenic, anti-inflammatory, and regenerative properties. Beyond its ability to stimulate stem or progenitor cell differentiation and migration, its role in regeneration is suggested by its presence in neural development, increased levels following neural injury, and its evolutionary conserved sequence.

Disc degeneration

Lumbar degenerative disease, which leads to lower back pain, is an endurance that aging man faces. Degenerative disc disease results from the loss by apoptosis of annulus cells, which make up the outer fibrous capsule of the intervertebral disk. A study conducted at three major medical institutions reported that Tβ4 significantly reduced disc cell apoptosis, suggesting a potential treatment for degenerative disc disease and chronic discogenic lower back pain.[56]

Sepsis and endotoxic shock

The overwhelming inflammatory response and influx of inflammatory cells directed against a systemic bacterial infection often results in massive vasodilation, increased capillary permeability, decreased systemic vascular resistance, and hypotension. Despite apparently effective antibiotic treatment for the infection, many patients die due to hypoxia, brought on by hypotension, and multiple organ dysfunction or both. As a consequence of this cascade of events in sepsis, cellular injury, necrosis, and death result in the release of large quantities of G-actin into the blood and extracellular fluid, overwhelming the actin-sequestering system. An LD_{50} dose of LPS administered to rats rapidly reduces Tβ4 levels in the blood. In healthy human volunteers given low doses of endotoxin and patients with the septic shock, endogenous Tβ4 levels are similarly reduced.[34] With deficit levels of Tβ4 in the blood, its ability to suppress the onslaught of inflammatory cytokines and chemokines and recruitment of inflammatory cells is compromised. However, when multiple administrations of Tβ4 are given to mice after challenge with a lethal dose of LPS, mortality rates and blood levels of inflammatory cytokines, eicosanoids, and other molecules, that are highly elevated following endotoxin administration, are significantly reduced.[34] As hypothesized, the rapid disappearance of Tβ4 in the blood following LPS administration or during septic shock suggests that Tβ4 may be involved in the early events leading to activation of the inflammatory cascade and later the clinical sequelae of sepsis. Tβ4 may have utility in the clinic in the treatment of septic shock.

Clinical application—respiratory disease/cystic fibrosis (CF)

Pulmonary drug delivery by inhalation is the preferred route of administration for respiratory disease; consequently, Tβ4 has been formulated as an aerosol solution (RGN-457) for direct delivery to the lung. Matched with the appropriate aerosol delivery device, RGN-457 could be directed to target various regions of the conducting airways.

CF is an autosomal recessive disorder and the most common congenital fatal lung disease in the world. The major clinical problem in this condition is the development of progressive bronchiectasis, and most patients succumb to respiratory or cardiopulmonary failure. Neutrophils are the predominant cells in the local inflammatory response to endobronchial bacterial infection in CF and

neutrophil-derived neutrophil elastase and other uninhibited proteases are found in the airways of these patients. Necrotic death of inflammatory and bacterial cells releases large amounts of actin and DNA, which copolymerize in CF sputum, contributing to the viscous purulent secretions in the airways that lead to reduced pulmonary function and exacerbations of infection. A pharmaceutical agent that can cause a disruption or destabilization of the actin-DNA network to allow for improved sputum clearance and suppression of the inflammatory response would be desirable. Tβ4 has been shown to reduce the cohesivity of CF sputum in a dose- and time-dependent manner. With DNase, Tβ4 significantly decreases sputum elasticity.[57] Along with its proven anti-inflammatory properties, ability to limit inflammatory cell recruitment, and proven safety for use as an inhaled drug (see Table 1), Tβ4 appears to be a most useful candidate for use in CF.

Summary

RegeneRx's Tβ4 is a synthetic copy of the naturally occurring, highly conserved 43–amino-acid peptide, found in most mammalian tissues and cells. It is the major G-actin-sequestering peptide in all eukaryotic cells and key actin regulator in mammals. Numerous nonclinical studies have defined Tβ4's safety profile and identified a range of functions and "moonlighting" activities, important for wound healing and repair and tissue regeneration. Those important qualities and features make Tβ4 an ideal candidate for treating injurious diseases and conditions in man involving, among other things, those of the skin, eye, heart, and brain. Clinical trials have established Tβ4's safety in man and provided direction for its continued use. On the horizon are plans to enter a most exciting time in the clinical development of Tβ4 when it is evaluated as a treatment for cardiac and neural repair indications.

Conflicts of interest

The authors declare no conflicts of interest.

References

1. Stoeva, S., S. Hörger & W. Voelter. 1997. A novel β-thymosin from the sea urchin: extending the phylogenetic distribution of β-thymosins from mammals to echinoderms. *J. Pept. Sci.* **3:** 282–290.
2. Goldstein, A.L., E. Hannappel & H.K. Kleinman. 2005. Thymosin β4: actin-sequestering protein moonlights to repair injured tissues. *Trends Mol. Med.* **11:** 421–429.
3. Huff, T., C.S.G. Muller, A.M. Otto, *et al*. 2001. β-Thymosins, small acetic peptides with multiple functions. *Int. J. Biochem. Cell Biol.* **33:** 205–220.
4. Grillon, C., K. Rieger, J. Bakala, *et al*. 1990. Involvement of thymosin β4 and endoproteinase Asp-N in the biosynthesis of the tetrapeptide AcSerAspLysPro a regulator of the hematopoietic system. *FEBS Lett.* **274:** 30–34.
5. Zabock, J., H. Oschkinat, E. Hannappel, *et al*. 1990. Solution conformation of thymosin β4: a nuclear magnetic resonance and simulated annealing study. *Biochemistry* **29:** 7814–7821.
6. Czisch, M., M. Schleicher, S. Horger, *et al*. 1993. Conformation of thymosin β4 in water determined by MNR spectroscopy. *Eur. J. Biochem./FEBS.* **218:** 335–344.
7. Domanski, M., M. Hertzog, J. Coutant, *et al*. 2004. Coupling of folding and binding of thymosin β4 upon interaction with monomeric actin monitored by nuclear magnetic resonance. *J. Biol. Chem.* **279:** 23637–23645.
8. Low, T.L. & A.L. Goldstein. 1982. Chemical characterization of thymosin beta 4. *J. Biol. Chem.* **257:** 1000–1006.
9. Hannappel, E. & M. van Kampen. 1987. Determination of Thymosin β4 in human blood cells and serum. *J. Chromatogr.* **397:** 279–285.
10. Hannappel, E. 2007. β-Thymosins. *Ann. N.Y. Acad. Sci.* **1112:** 21–37.
11. Grant, D.S., J.L. Kinsella, M.C. Kibbey, *et al*. 1995. Matrigel induces thymosin β4 gene in differentiating endothelial cells. *J. Cell Sci.* **108:** 3685–3694.
12. Sanders, M.C. *et al*. 1992. Thymosin β4 (Fx peptide) is a potent regulator of actin polymerization in living cells. *Proc. Natl. Acad. Sci. USA* **89:** 4678–4682.
13. Malinda, K.M. *et al*. 1997. Thymosin β4 stimulates directional migration of human umbilical vein endothelial cells. *FASEB J*. **11:** 474–481.
14. Sosne, G. *et al*. 2004. Thymosin β4 stimulates laminin-5 production independent of TGF-beta. *Exp. Cell Res.* **293:** 175–183.
15. Hormia, M. *et al*. 1995. Rapid spreading and mature hemidesmosome formation in HaCaT keratinocytes induced by incubation with soluble laminin-5r. *J. Invest. Dermatol.* **105:** 557–561.
16. Larjava, H. *et al*. 1993. Expression of integrins and basement membrane components by wound keratinocytes. *J. Clin. Invest.* **92:** 1425–1435.
17. Bock-Marquette, I., A. Saxena, M.D. White, *et al*. 2004. Thymosin β4 activates integrin-linked kinase and

promotes cardiac cell migration, survival and cardiac repair. *Nature* **432:** 466–472.
18. Grant, D.S., W. Rose, C. Yaen, *et al*. 1999. Exogenous Thymosin β4 enhances endothelial differentiation and angiogenesis. *Angiogenesis* **3:** 125–135.
19. Koutrafouri, V. *et al*. 2001. Effect of thymosin peptides on the chick chorioallantoic membrane angiogenesis model. *Biochim. Biophys. Acta* **1568:** 60–66.
20. Cha, H.K., M.J. Jeong, & H.K. Kleinman. 2003. Role of thymosin beta 4 in tumor metastasis and angiogenesis. *J. Natl. Cancer Inst.* **95:** 1674–1680.
21. Philp, D., T. Huff, Y.S. Gho, *et al*. 2003. The actin binding site on thymosin β4 promotes angiogenesis. *FASEB J*. **17:** 2103–2105.
22. Van Troys, M. *et al*. 1996. The actin binding site of thymosin beta 4 mapped by mutational analysis. *EMBO J*. **15:** 201–210.
23. Smart, N., C.A. Riseboro, A.A. Melville, *et al*. 2007. Thymosin β4: induces adult epicardial progenitor mobilization and neovascularization. *Nature* **445:** 177–182.
24. Philp, D., A.L. Goldstein & H.K. Kleinman. 2004. Thymosin beta 4 promotes angiogenesis, wound healing, and hair follicle development. *Mech. Ageing Dev.* **125:** 113–115.
25. Philp, D., M. Nguyen, B. Scheremeta, *et al*. 2004. Thymosin increases hair growth by the activation of clonogenic hair follicle stem cells. *FASEB J*. **18:** 385–387.
26. Sosne, G., A. Siddiqi & M. Kurpakus-Wheater. 2004. Thymosin-β4 Inhibits Corneal epithelial cell apoptosis after ethanol exposure. *Invest. Ophthalmol. Vis. Sci.* **45:** 1095–1100.
27. Kupatt, C., R. Hinkel, M. Lamparter, *et al*. 2005. Retroinfusion of embryonic endothelial progenitor cells attenuates ischemia-reperfusion injury in pigs: role of phosphatidylinositol 3-kinase/AKT kinase. *Circulation* **112**(Suppl.): I-117–I-122.
28. Hinkel, R., C. El-Aouni, T. Olson, *et al*. 2008. Thymosin β4 Is an essential paracrine factor of embryonic endothelial progenitor cell-mediated cardioprotection. *Circulation* **117:** 2232–2240.
29. Bock-Marquette, I., S. Shrivastava, G.C. Teg Pipes, *et al*. 2009. Thymosin β4 mediated PKC activation is essential to initiate the embryonic coronary developmental program and epicardial progenitor cell activation in adult mice in vivo. *J. Mol. Cell. Cardiol.* **46**(5): 728–738.
30. Malinda, K.M., G.S. Sidhu, K. Mani, *et al*. 1999. Thymosin beta4 accelerates wound healing. *J. Invest. Dermatol*. **113:** 364–368.
31. Sosne, G., I. Christopherson, R.P. Barrett, *et al*. 2005. Thymosin β4: modulates corneal matrix metalloproteinase levels and polymorphonuclear cell infiltration after alkali injury. *Invest. Ophthalmol. Vis. Sci*. **46:** 2388–2394.
32. Sosne, G., E.A. Szliter, R. Barrett, *et al*. 2002. Thymosin β4: promotes corneal wound healing and decreases inflammation *in vivo* following alkali injury. *Exp. Eye Res*. **74:** 293–299.
33. Sosne, G., C.C. Chan, K. Thai, *et al*. 2001. Thymosin beta 4 promotes corneal wound healing and modulates inflammatory mediators in vivo. *Exp. Eye Res.* **72:** 605–608.
34. Badamchian, M., M.O. Fagarasan, R.L. Danne, *et al*. 2003. Thymosin beta 4 reduces lethality and down-regulates inflammatory mediators in endotoxin-induced septic shock. *Int. J. Immunopharmacol*. **3:** 1225–1233.
35. Sosne, G., P. Qiu, P.L. Christopherson, *et al*. 2007. Thymosin beta 4 suppression of corneal NFkappaB: a potential anti-inflammatory pathway. *Exp. Eye Res.* **48:** 663–669.
36. Philp, D., M. Badamchian, B. Scheremeta, *et al*. 2003. Thymosin beta 4 and a synthetic peptide containing its actin-binding domain promote dermal wound repair in db/db diabetic mice and in aged mice. *Wound Rep. Reg.* **11:** 19–24.
37. Fine, J.-D. 2007. Epidermolysis bullosa: a genetic disease of altered cell adhesion and wound healing, and the possible clinical utility of topically applied thymosin β4. *Ann. N.Y. Acad. Sci.* **1112:** 396–406.
38. U.S. Department of Health and Human Services, Food and Drug Administration 2006. Guidance for industry: chronic cutaneous ulcer and burn wounds – developing products for treatment.
39. Dermal wounds: pressure sores: philosophy of the IAET 1988. *J. Enterostomal Ther.* **15:** 4–17.
40. Ennis, J.E. & P. Menesis. 2003. Standard, appropriate and advanced care and medical-legal considerations: part two – venous ulcerations. *Wounds* **15:** 107–122.
41. Kerstein, M.D. & V. Gahtan. 1998. Outcomes of venous ulcer care: results of longitudinal study. *Ostomy/Wound Manage*. **44:** 52–64.
42. Falanga, V., D. Margolis, O. Alvarez, *et al*. 1998. Rapid healing of venous ulcers and lack of clinical rejection with allogenic cultured human skin equivalent. *Arch. Dermatol.* **134:** 283–300.
43. Lyon, R.T., F.J. Veith, L. Bolton, *et al*. 1998. Clinical benchmark for healing chronic venous ulcers. *Am. J. Surg.* **176:** 172–175.
44. Dunn, S.P., D.G. Heidemann, C.Y.C. Chow, *et al*. 2009. Treatment of chronic non-healing neurotrophic corneal

epithelial defects with thymosin beta 4. *Arch. Ophthalmol.* In press.
45. Crockford, D. 2007. Development of thymosin β4 for the treatment of patients with ischemic heart disease. *Ann. N.Y. Acad. Sci.* **1112:** 385–395.
46. Sun, W. & H. Lim. 2007. Neurotrophic roles of the beta-thymosins in the development and regeneration of the nervous system. *Ann. N.Y. Acad. Sci.* **1112:** 210–218.
47. van Kesteren, R.E., C. Carter, H.M. Dissel, *et al*. 2006. Local synthesis of actin-binding protein beta-thymosin regulates neurite outgrowth. *J. Neurosci.* **26:** 152–157.
48. Yang, H., X. Cheng, Q. Yao, *et al*. 2008. The promotive effects of thymosin beta4 on neuronal survival and neurite outgrowth by upregulating L1 expression. *Neurochem. Res.* **33:** 2269–2280.
49. Popoli, P., R. Pepponi, A. Martire, *et al*. 2007. Neuroprotective Effects of Thymosin β4 in Experimental Models of Excitotoxicity. *Ann. N.Y. Acad. Sci.* **1112:** 219–224.
50. Zhang, J., Z.G. Zhang, D. Morris, *et al*. 2009. Neurological functional recovery after thymosin beta 4 treatment in mice with experimental autoencephalomyelitis. *Neuroscience*. In press.
51. Morris, D., M. Chopp, L. Zhang, *et al*. 2009. Thymosin β4 improves functional outcome in a rat model of embolic stroke. *J. Cereb. Blood Flow Metab*. In press.
52. Hulbert, R.J. 2000. Methylprednisolone for acute spinal cord injury: an inappropriate standard of care. *J. Neurosurg.* **93**(1 Suppl.): 1–7.
53. Carlson, S.L., M.E. Parrish, J.E. Springer, *et al*. 1998. Acute inflammatory response in spinal cord following impact injury. *Exp. Neurol.* **151**(1): 77–88.
54. Colby, G.P., Y.J. Sung & R.T. Amron. 2005. mRNAs encoding the Aplysia homologues of fasciclin-I and beta-thymosin are expressed only in the second phase of nerve injury and are differentially segregated in axons regenerating in vitro and in vivo. *J. Neurosci. Res.* **82:** 484–498.
55. Crowe, M.J., J.C. Bresnahan, S.L. Shuman, *et al*. Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys. *Nat. Med.* **3**(1): 73–76.
56. Tapp, H., R. Deepe, J.A. Ingram, *et al*. 2009. Exogenous thymosin beta (4) prevents apoptosis in human intervertebral annulus cells in vitro. *Biotech. Histochem.* **25:** 1–8.
57. Kater, A., M.O. Henke & B.K. Rubin. 2007. The role of DNA and actin polymers on the polymer structure and rheology of cystic fibrosis sputum and depolymerization by gelsolin or thymosin beta 4. *Ann. N.Y. Acad. Sci.* **1112:** 140–153.