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## REPORT

## Thymosin beta 4 and Skin Repair

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The promise of repairing sun parched aging skin is alluring, especially if damage control may be attained by applying a substance that is abundant in our body. Thymosin beta 4 (Tb4), a molecule that accelerates wound healing in animals and cultured cells, "may be valuable in repairing skin damage caused by sun or even by the wear and tear of aging?" This hopeful message of Tb4's potential to restore damaged human skin was voiced at the 5th International Symposium on Aging Skin, in California (May 2001), by Dr. Allan Goldstein, Chairman of the Biochemistry Department at George Washington University and founder of RegeneRX Biopharmaceuticals. RegeneRX is carrying out preclinical research on Tb4 as a wound healer, in collaboration with scientists at the National Institutes of Health.



Skin is the largest organ of the body, which makes up 16% of total body weight. It is also the largest organ that provides immune protection and plays a role in inflammation. Composed of specialized epithelial and connective tissue cells, skin is our major interface with the environment, a shield from the outside world and a means of interacting with it. As such, the skin is subjected to insults and injuries: burns from the sun's ultraviolet radiation that elicit inflammatory reactions, damage from environmental pollutants and wear and tear that comes with aging.



There are several layers in the skin; the outer epidermis and beneath it the dermis and the subcutaneous layer. Cells in the epidermis include keratinocytes, its major cell type, that move continuously from the lower basal layer where they are formed by cell division. Other cells in the epidermis are the melanocytes that synthesize pigment and transfer it to the keratinocytes, giving our skin its color, and a wide variety of immune cells that maintain immune surveillance and secrete substances called cytokines, like interleukin 1 and 2, which are active in inflammation. The dermis contains connective tissue, mainly collagen, blood vessels, various types of immune white cells and fibroblasts.



The structure that provides the cell with form is the cytoskeleton, whose protein actin, a housekeeping molecule in cells, comprises 10% of the cell protein. Actin is essential for cell division, cell movement, phagocytosis (engulfing foreign bodies in immunoprotection) and differentiation.



Cells on the surface of the skin are constantly being replaced by regeneration from below. The repair of a wound is a scaling up of this normal process, with additional complex interactions among cells, formation of new blood vessels, collagen, more extensive cell division and cell migration, as well as strict control of inflammatory cells and the cytokines they release to resolve the inflammation.

Skin damage and aging are induced to a large extent by free radicals from the sun and environmental pollutants and from oxidants produced during infection and inflammation. Lipid peroxidation of membranes and increased inflammatory substances, such as thromboxanes and leukotriens, add insult to injury. While skin damage accumulates with age, repair processes slow down. Thus, any boost by a molecule that would reduce free radicals and accelerate molecular events in healing has the potential to hasten skin repair. Tb4 has such healing qualities.

The nature of Tb4

An effective healer, Tb4 can be administered topically on the surface of cells and

systemically, through injection. Besides healing skin wounds, Tb4 has been shown to promote repair in the cornea of the eye, in rats, thus preventing loss of vision.

Thymosin beta 4 is a small 43 amino acid protein (a peptide) that was originally identified in calf thymus, an organ that is central in the development of immunity. Tb4 was later found in all cells except red blood cells. It is highest in blood platelets that are the first to enter injured areas, in wound healing. Tb4 is also detected outside cells, in blood plasma and in wound and blister fluids.

Its unique potential as a healing substance lies in that it interacts with cellular actin and regulates its activity. Tb4 prevents actin from assembling (polymerizing) to form filaments but supplies a pool of actin monomers (unpolymerized actin) when a cell needs filaments

for its activity. A cell cannot divide if actin is polymerized. Tb4 therefore serves in vivo to maintain a reservoir of unpolymerized actin that will be put to use when cells divide, move and differentiate.

Tb4 has other effects that are needed in healing and repair of damaged tissue. It is a chemo-attractant for cells, stimulates new blood vessel growth (angiogenesis), downregulates cytokines and reduces inflammation, thus protecting newly formed tissue from damaging inflammatory events. Tb4 has been shown to reduce free radical levels (with similar efficiency as superoxide dismutase), decrease lipid peroxidation, inhibit interleukin 1 and other cytokines, and decrease inflammatory thromboxane (TxB2) and prostaglandin (PGF2 alpha).

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### Wound healing

A critical step in wound healing is angiogenesis. New vessels are needed to supply nutrients and oxygen to the cells involved in repair, to remove toxic materials and debris of dead cells and generate optimal conditions for new tissue formation. Another important step is the directional migration of cells into the injured area, joining up to repair the wound. This requires an attractant that will direct the cells to the wound and propel them to the site. These critical steps in wound healing are regulated by beta 4, as seen in the following experiments.

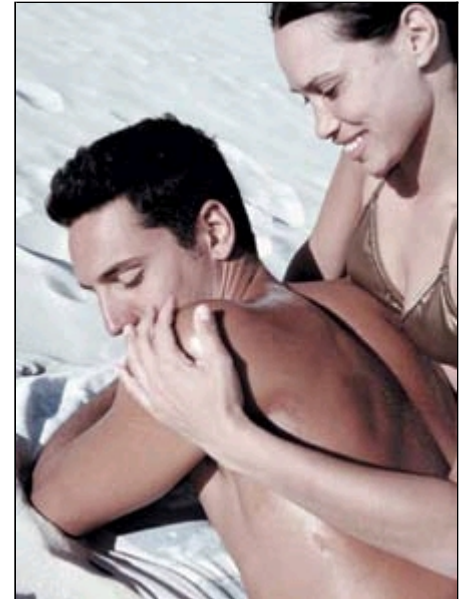
### Endothelial cells

Cells that line blood vessels (endothelial cells), taken from human umbilical chord veins, were grown in culture and the layer of cells subjected to a scratch wound. Cultures were then treated with Tb4 or kept in growth medium without Tb4. When examined four hours later, Tb4 treatment attracted cells to migrate into the wound and accelerated their movement, showing it is a chemoattractant. Cell migration was four to six times faster in the presence of Tb4 compared to the migration of untreated cells. Tb4 also hastened wound closure and increased the production of enzymes, called metalloproteases, that could pave the way for angiogenesis by breaking down barrier membranes and facilitating the invasion of new cells to the needy area, to form new vessels. Other experiments showed Tb4 acts in vivo. When endothelial cells were implanted under the skin in a gel supplemented with Tb4, the cells formed vessel-like structures containing red blood cells, indicating the ability to stimulate angiogenesis in the animals.

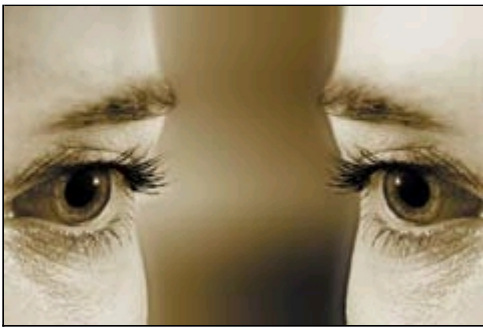
### Skin repair

Thymosin beta 4 accelerated skin wound healing in a rat model of a full thickness wound where the epithelial layer was destroyed. When Tb4 was applied topically to the wound or injected into the animal, epithelial layer restoration in the wound was increased 42% by day four and 61% by day seven, after treatment, compared to untreated. Furthermore, Tb4 stimulated collagen deposition in the wound and angiogenesis. Tb4 accelerated keratinocyte migration, resulting in the wound contracting by more than 11%, compared to untreated wounds, to close the skin gap in the wound. An analysis of skin sections (histological observations) showed that the Tb4 treated wounds healed faster than the untreated. Proof of accelerated cell migration was also seen in vitro, where Tb4 increased keratinocyte migration two to three fold, within four to five hours after treatment, compared to untreated keratinocytes.

### Repair of the cornea



The promise of repairing sun parched aging skin is alluring, especially if damage control may be attained by applying a substance that is abundant in our body.



The cornea is the outer thin layer of epithelial cells protecting the eye. After wounding, timely resurfacing of the cornea with new cells is critical, to prevent loss of normal function and loss of vision. Corneal epithelial healing occurs in stages, with cells migrating, dividing and differentiating. Therapies for corneal injury are limited. Therefore, the recent finding that Tb4 promotes corneal wound repair in animal models offers hope for a therapeutic product that will improve the clinical outcome of patients with injured corneas.

In the experiments, an epithelial wound was made in the corneas of sedated rats. A Tb4 solution was applied at several concentrations to the injured eyes in one group of rats while another group was treated with a solution without Tb4. Following 12, 24 and 36 hours, the eyes were tested by microscopic observation for epithelial growth over the injured site. Investigators found the Tb4 accelerated corneal wound repair at doses of Tb4 similar to those found to repair skin wounds. When tested 24 hours after treatment, the rate of accelerated repair was proportional to the concentration of Tb4, with the highest dose (25 microgram) showing a threefold acceleration of epithelial cell migration, compared to untreated. Treatment with Tb4 showed anti-inflammatory effects, helping resolve the injury. An application to human cells in a model of human corneal cells in culture showed that Tb4 enhanced epithelial cell migration in vitro.

#### RegeRx and Tb4

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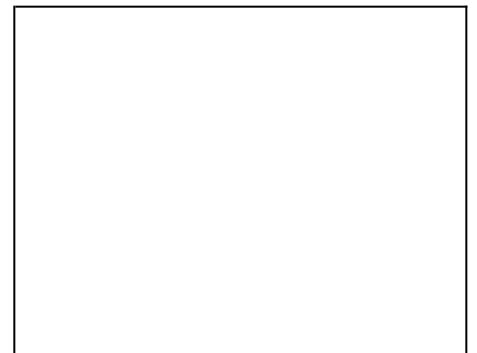
Thymosin beta 4, developed by RegeneRx Biopharmaceuticals as a pharmaceutical for the healing of wounds, is a synthetic version of the natural peptide. As Dr. Allan Goldstein emphasizes, "Tb4 represents a new class of wound healing compounds. It is not a growth factor or cytokine, but rather exhibits a number of physiological properties which include the ability to sequester and regulate actin, its potent chemotactic properties. . . and its capability to downregulate a number of inflammatory cytokines that are present in chronic wounds." When a wound heals there are many growth factors produced in the area so that additional factors, such as those currently on the market for wound healing, may help but are not necessarily lacking. Tb4 treatment, however, adds a new dimension to wound repair by providing cells with actin as needed, for cell migration, replication and differentiation.

RegeneRX Biopharmaceuticals is focusing on the commercialization of Tb4 "For the treatment of injured tissue and non-healing wounds, to enable more rapid repair and/or tissue regeneration." Especially needy are diabetics who suffer from poor blood circulation and loss of sensation of pain that keeps their wounds unnoticed and unattended for days, leading to ulcers that may not heal. Other hard healing wounds are pressure ulcers in patients who are bed ridden and often receive skin grafts as treatment, or reconstructive surgery.

RegeneRx is continuing with pre-clinical research, in collaborative arrangements with the National Institutes of Health, accumulating data on the effects of Tb4 and aiming for an IND application (Investigational New drug App-lication) to proceed with clinical studies. Phase I clinical trials will determine the ability of Tb4 to repair ulcers in diabetic patients and to reduce inflammation and accelerate recovery from burns and abrasions to the cornea.

#### Aging skin

The potential of Tb4 to repair sun damaged and aging skin is yet to be established by extensive studies. Many of the biological events that occur in wounding are involved in skin impaired by sun and aging. Ultraviolet radiation damage or other injuries to skin that are associated with aging may be in the future repairable with Tb4, similar to the success with wound repair. It is a hopeful prediction that this small anti-inflammatory molecule, which plays a vital role in regeneration, remodeling and healing of damaged tissues, would help rejuvenate aging skin. The effects of Tb4 in accelerating wound repair are important following surgery; Tb4 would then have practical applications following cosmetic surgery, a procedure growing in popularity in our society, in dealing with aging skin.



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