Thymosin β_4 increases hair growth by activation of hair follicle stem cells[1]

DEBORAH PHILP, MYCHI NGUYEN,* BROOKE SCHEREMETA,† SHARLEEN ST.-SURIN,‡ ANA M. VILLA,§ ADAM ORGEL, HYNDA K. KLEINMAN,[2] AND MICHAEL ELKIN††**

Cell Biology Section, NIDCR, National Institutes of Health (NIH), Bethesda, Maryland, USA; *Drexel University, Philadelphia, Pennsylvania, USA; †NIAMS, NIH, Bethesda, Maryland, USA; ‡10123 Prince Place, Upper Marlboro, Maryland, USA; §University of Maryland, Baltimore, Maryland, USA; **University of Maryland, Rockville, Maryland, USA; and ††Tumor Biology Unit, Department of Oncology, Hadassah-Hebrew University Hospital, Jerusalem 91120 Israel

SPECIFIC AIMS

We have explored the ability of thymosin β_4 to promote hair growth in normal rats and mice and determined the mechanism by which thymosin β_4 acts to promote hair growth by examining its effects on follicle stem cell growth, migration, differentiation, and protease production.

PRINCIPAL FINDINGS

1. Thymosin β_4 promotes hair growth in normal rats and mice

While studying wound healing in rat skin, we unexpectedly observed at the histological level increased hair growth at the wound margins 7 days after topical treatment with thymosin β_4. We shaved the skin of healthy rats and applied thymosin β_4 topically on one side of the shaved area and the control vehicle on the opposing lateral side of the same animal. After 7 days of treatment, we observed an increased number of anagen-phase hair follicles in skin areas treated with thymosin β_4 (**Fig. 1*a, d***). The number of anagen follicles was ~twofold greater than in rats treated with vehicle alone. The increased number of hairs in anagen phase was retained with triweekly treatment over 30 days. Within 14 days of treatment cessation, the number of active hair follicles decreased to control levels**.** We next tested whether thymosin β_4 would promote hair growth in 8-wk-old C57BL6 wild-type mice. Mice used in this experiment had all their hair follicles in the telogen stage as judged by the pink skin color**.** The mice were shaved and thymosin β_4 was applied topically on the shaved area. Control animals were treated with vehicle alone. As shown in Fig. 1*b, e*, thymosin β_4-treated (but not control) animals displayed quick hair regrowth. Histological examination confirmed thymosin β_4-induced activation of the hair follicles (Fig. 1*b, e*). Hair was grossly visible in the treated mice (Fig. 1*c, f*).

2. Thymosin β_4 protein expression by a subset of hair follicle stem cells

We first explored the spatial and temporal pattern of endogenous thymosin β_4 expression in hair follicles during depilation-induced, synchronized adult hair cycling in C57BL/6J mice. We wanted to correlate the observed effects of thymosin β_4 administration with possible functional involvement of endogenous thymosin β_4 in hair growth. Low levels of thymosin β_4 protein were observed in follicles at the telogen (resting) phase before depilation. Thymosin β_4 expression was confined to a small number of cells residing in the bulge region at the level of the insertion of the arrector pili muscle. Hair follicle transition to early anagen (day 4 after depilation) was associated with an increased number of thymosin β_4-expressing cells in the bulge region. At late anagen (day 9 after depilation), a significant number of cells located in the lower follicle (matrix surrounding part of the outer root sheath) expressed thymosin β_4. The sebaceous gland was stained at all stages due to nonspecific absorption, as found by others with different antibodies. Thus, with the progression of hair growth cycle, thymosin β_4-positive cells initially detected only in the bulge were observed at the bulb area, suggesting they are migrating from the bulge region. These data show that the temporal and spatial distribution of thymosin β_4-expressing cells was similar to the pattern proposed for the hair follicle stem cells and their daughter cells, i.e., emanating from the bulge and migrating downward to give rise to matrix cells that subsequently generate the hair shaft.

[1] To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.03-0244fje; doi: 10.1096/fj.03-0244fje

[2] Correspondence: Cell Biology Section, NIH, NIDCR, Bldg. 30, Room 433, 30 Convent Dr., MSC 4370, Bethesda, MD 20892, USA. E-mail: hkleinman@dir.nidcr.nih.gov

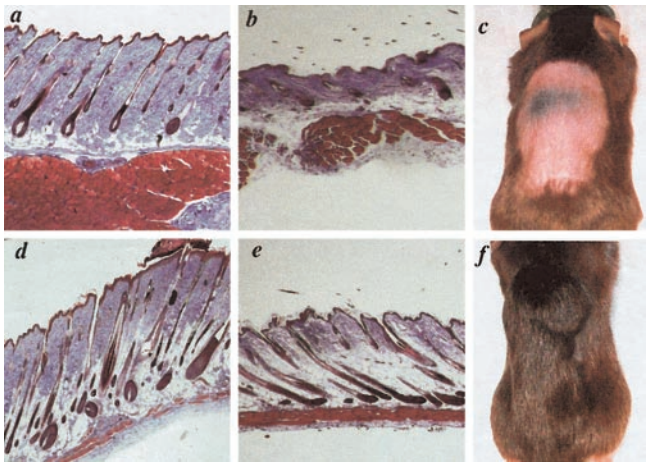

Figure 1. Histological and gross appearance of skin from control and thymosin β_4-treated rats and mice after depilation-induced hair growth in C57BL/6 mice. Hair growth in normal rats and mice. Animals (3–5/group) were treated on one side with vehicle and on the other side with thymosin β_4; for the mouse studies (2/group), mice were used for treatment and control. All sections were stained with Masson's trichrome. Microphotographs of the rat and mouse histological sections were made at ×32 and ×66, respectively. *a)* Control vehicle-treated rat skin after 7 days. *b)* Control vehicle-treated mouse skin after 28 days. *c)* Gross appearance of control vehicle-treated mouse skin after 28 days. *d)* Rat skin after 7 days of thymosin β_4 treatment. *e)* Mouse skin after 28 days of thymosin β_4 treatment. *f)* Gross appearance of mice after 28 days of thymosin β_4 treatment.

3. Cultured rat vibrissa clonogenic keratinocytes express thymosin β_4

We studied rat vibrissae follicle keratinocytes from the bulge region to determine whether isolated stem cells express thymosin β_4. Hair follicle stem cells have been identified as bulge-residing keratinocytes with a high in vitro clonogenic potential. Although hair follicle stem cells are not fully characterized, they preferentially express keratin 15 (K15). We isolated clonogenic keratinocytes from the rat vibrissa bulge region and found that the isolated cells were highly clonogenic. These cells were positive for the stem cell marker keratin 15 as well as for keratins 5, 6, and 14, known to be expressed by bulge stem cells (data not shown). These cells lacked keratin 10, an early marker of terminal keratinocyte differentiation (data not shown). When cultured in vitro, these cells were able to move with an average velocity of 0.43 μm/min. We conclude that the obtained cell population represents the immediate progeny of hair follicle stem cells. We found that these cells expressed thymosin β_4 after 7–10 days of culturing in vitro. Treatment of the clonogenic keratinocytes with exogenous thymosin β_4 caused a dose-dependent decrease in the expression levels of the multipotent undifferentiated stem cell marker K15 (**Fig. 2**). A decline in K15 is associated with stem cell differentiation. We found that thymosin β_4 had no effect on stem cell proliferation (data not shown). These data indicate that the clonogenic keratinocytes isolated from rat vibrissa bulge represent the stem cell population and suggest that thymosin β_4 is important for early stem cell differentiation.

4. Thymosin β_4 promotes the migration of hair clonogenic keratinocytes in vitro

Thymosin β_4 has been shown to promote endothelial cell migration. We found that cultured clonogenic keratinocytes migrate to thymosin β_4 after 4.5 h in Boyden chamber assays. In the presence of thymosin β_4, cell migration was increased almost twofold (69.0±7.1 vs. 113.3±5.5 cell number/field, $P \leq 0.001$) at 1 ng over migration in the presence of medium containing vehicle alone (negative control). The effect of thymosin β_4 on cell migration was greatest at 1 ng/mL; at 100 and 1000 ng/mL migration was decreased. Earlier we found that 1 ng/mL was potent for endothelial and keratinocyte migration.

5. Thymosin β_4 augments the production and secretion of matrix metalloproteinase 2 (MMP-2) by clonogenic keratinocytes

Enzymatic degradation and remodeling of the extracellular matrix are necessary in normal hair development and growth**.** We have examined the effect of thymosin β_4 on the enzymatic activity of matrix metalloproteinases (MMPs), enzymes responsible for degradation of

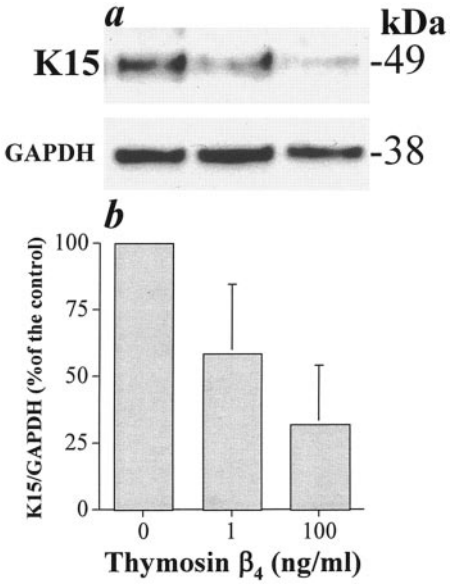

Figure 2. Exogenous thymosin β_4 decreases expression of keratin 15 in clonogenic keratinocytes from the rat vibrissa bulge region. Western blot analysis with anti-K15 antibody. Rat vibrissa clonogenic keratinocytes isolated from the bulge region were kept in the serum-free medium for 3 h, then incubated for 8 h in the absence or presence of thymosin β_4. The cells were washed with PBS and lysed in RIPA buffer. Protein concentration was determined and lysates normalized for equal protein were analyzed by Western blot with antibodies against keratin 15. The same membranes were reprobed with anti-GAPDH antibody.

major extracellular matrix proteins and therefore matrix and basement membrane remodeling in many biological processes, including hair follicle development and growth. Treatment of the clonogenic keratinocytes with exogenous thymosin β_4 caused almost a twofold increase in MMP-2. The increase in the levels of secreted and cell-derived enzyme MMP-2 was dose dependent. MMP-2 degrades collagen type IV and laminin, key proteins of the basement membrane. This effect on MMP-2 was specific, since the level of another collagen IV-degrading enzyme, MMP-9, was unchanged during thymosin β_4 treatment. Since MMP-2 plays a role in hair growth-associated extracellular matrix remodeling and cell migration, our data suggest that this enzyme may be a downstream effector through which thymosin β_4 exerts its effect on hair growth.

CONCLUSIONS AND SIGNIFICANCE

Thymosin β_4 is a 4.9 kDa molecule that functions as a major actin-sequestering protein in cells. It is up-regulated during endothelial cell differentiation; when added exogenously, it promotes endothelial cell differentiation and migration. In vivo, it promotes wound repair and is a potent anti-inflammatory agent. A related family member, thymosin β_{15}, is also important in the metastasis of certain tumor types. It was reported that thymosin β_4 exerts its effects on cell locomotion through specific interactions with actin that regulate cytoskeletal organization. We show that exogenously delivered thymosin β_4 promotes hair growth in normal rats and mice. When examining the distribution of endogenous thymosin β_4 through sequential phases of depilation-induced hair growth, we found that in the resting (telogen) follicle it is expressed in the small number of cells originating in the bulge region of the outer root sheath. As the follicles enter active growth phase (anagen), the subset of thymosin β_4-expressing cells in the outer root sheath is expanded toward the base of the follicle. At the peak of anagen, a significant number of thymosin β_4-expressing cells are found in the bulb area, in the outer root sheath, and among the hair matrix cells. Isolated clonogenic hair follicle keratinocytes, closely related (if not identical) to the hair follicle stem cells, produce thymosin β_4 when cultured in vitro for 7–10 days. The presence of exogenous thymosin β_4 caused a dose-dependent decrease in the expression of the stem cell marker K15 by clonogenic keratinocytes, suggesting that thymosin β_4 may promote early stem cell differentiation (i.e., transition to the transi-amplifying cell phenotype). Most important, treatment of the bulge-derived clonogenic keratinocytes with exogenous thymosin β_4 increased migration and production of MMP-2.

A critical step in the hair growth cycle is the movement of some of the bulge-residing stem cells downward, where their differentiated progeny contribute to complete regrowth or regeneration of the lower, cycling portion of the follicle. Our data indicate that thymosin β_4 facilitates this movement of the stem cells and their immediate progeny and, thus, exerts its promoting effect on hair growth (**Fig. 3**). The effect of thymosin β_4 on MMP-2 expression appears to play an important role in this system. MMP-2 has been shown to contribute to cellular migration by degrading extracellular matrix barriers for cell movement and through direct effects on cell locomotion in vitro. MMP-2 is involved in hair cycle-associated remodeling of the basement membrane, the specialized extracellular matrix structure surrounding the epithelial core of the follicle. Basement membrane remodeling is necessary for signaling between epithelial and stromal elements of the growing follicle and for elongation and invasion of the lower follicle into subcutaneous tissue during the anagen phase.

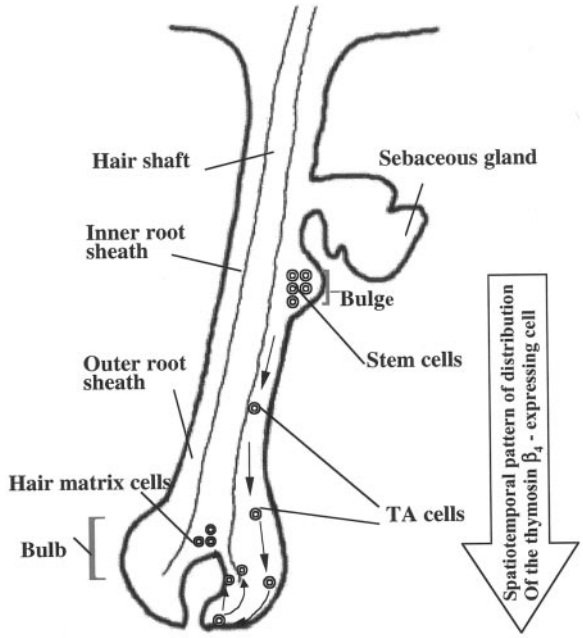

Figure 3. Schematic effects of thymosin β_4 on stem cells in hair follicles.

Hair growth acceleration by thymosin β_4 may also be attributed to proangiogenic and other biological activities of this molecule. It was recently reported that VEGF promotes hair follicle development presumably due to its angiogenic activity. Thymosin β_4 is angiogenic (like VEGF), and the activity of thymosin β_4 may be due to its angiogenic activity. Another angiogenic molecule, hepatocyte growth factor, has been found to promote hair growth. Hepatocyte growth factor up-regulates thymosin β_4 expression and may be acting by increasing thymosin β_4 and/or synergizing with it. Steroids have been used to treat certain types of hair loss. Thymosin β_4 is the anti-inflammatory molecule identified as increased in steroid-treated monocytes. Thus, treatment with steroids may involve the activity of thymosin β_4 on hair growth.

Taken together, our results suggest that thymosin β_4 exerts a profound hair-promoting effect through a combined action on several critical events in hair follicle growth such as stem cell migration, ECM-degrading enzyme production, and differentiation. FJ