Topical Thyroid Hormone Accelerates Wound Healing in Mice

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Although the physiologic role of thyroid hormone in skin is not well understood, mounting evidence suggests that T₃ plays an important role in epidermal proliferation. The goal of this project was to evaluate whether the topical application of supraphysiologic doses of T₃ could accelerate wound healing. We evaluated mice treated with topical T₃ vs. the same mice receiving vehicle alone (Novasome A). Ten-millimeter diameter (79 mm²) dorsal skin wounds were established in all animals, and wounds were remeasured 4 d after injury. All animals were evaluated twice: once with the T₃ treatment and once with the vehicle alone. Daily topical application of 150 ng T₃ resulted in 58% greater wound closure relative to wounds on the same animals receiving vehicle alone (P < 0.001). Furthermore, we determined that wound healing-associated keratin 6 protein expression in hair follicle keratinocytes increased in a dose-dependent manner in vivo during topical T₃ treatment. The data support our previous hypothesis that T₃ is necessary for optimal wound healing. Now, we further suggest that topical thyroid hormone may be an inexpensive agent to hasten healing of certain wounds. (Endocrinology 146: 4425–4430, 2005)

HYPOTHYROID HORMONE IS essential for optimal epidermal cell proliferation (1–4). In vitro, T₃ stimulates keratinocyte proliferation. In vivo, topical T₃ stimulates epidermal proliferation, dermal thickening, and hair growth (3, 5, 6). Topical triac (tri-iodothyroacetic acid) thickens skin by stimulating production of collagen (7, 8).

T₃ effects on skin depend on route of delivery. In contrast to the effects seen with topical T₃, systemically induced thyrotoxicosis in rodents results in the skin thinning and hair loss traditionally associated with excess T₃ (3) along with associated collagen loss (9).

Recently, we determined that hypothyroidism in mice retarded wound healing 2-fold relative to euthyroidism (10). Prior reported effects of thyroid hormone on wound healing had been contradictory. Lennox and Johnston (11) reported that exogenous T₃ given systemically improved the rate of wound healing in rats as well as the strength of the scars. They further reported decelerated wound healing in hypothyroid rats. Mehregan and Zamick (12, 13) also reported that additional thyroid hormone stimulated the rate and quality of wound healing in euthyroid rats. Scars were smoother in animals receiving T₃ in drinking water. There are several reports that hypothyroid patients required thyroid hormone to achieve healing of radiation-induced neck fistulae (14–16).

Conversely, Pirk et al. (17) noted no change in wound healing with euthyroid hamsters receiving ip T₃. Cannon (18) reported that hypothyroidism did not diminish wound strength in pigs, and Ladenson et al. (19) failed to detect wound healing deficits in hypothyroid humans.

The keratin genes encode the intermediate filaments, making up about 30% of the protein of the epidermis. Some associations between the keratin genes and specific phases of skin growth have been made (20–22), including the following: keratins 1 and 10 are associated with epidermal differentiation; keratins 6, 16, and 17 are associated with epidermal proliferation and wound repair; and keratins 5 and 14 are expressed in the basal skin layer, their expression decreased as the skin cells differentiate. In keratin 6 (K6) knockout mice, the absence of K6 resulted in diminished superficial wound healing (23).

Although thyroid hormone stimulates gene expression of keratins 6, 16, and 17 (10), to date only negative thyroid hormone response elements have been identified for the keratin genes associated with proliferation (24, 25).

Investigators have recognized a role for follicular keratinocytes in wound healing (26). Recent data suggest that hair follicular cells are integral to wound healing with outer route sheath cells migrating to become epidermis and dermal sheath cells becoming the primary wound healing fibroblasts (27).

The goal of this project was to evaluate whether the topical application of supra-physiologic doses of T₃ could accelerate wound healing and whether epidermal K6 protein expression would be altered by topical thyroid hormone.

Materials and Methods

Preparation of topical thyroid hormone cream

We prepared a topical T₃ cream by mixing T₃ (Sigma, St. Louis, MO) into a liposome vehicle (Novasome A, IGI Inc., Buena, NJ) and applying T₃ in 30 µl vehicle daily to a defined, shaved 3 × 3-cm region in the midline backs of 5-wk-old female CD-1 mice (Charles River, Boston, MA).

In vivo wound healing protocol

All animal experimentation described was conducted in accord with accepted standards of humane animal care in a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Boston University School of Medicine.
All mice were anesthetized with 3 × 3-cm midline areas of their backs delineated and shaved. Four 10-mm diameter (79 mm²) full thickness wounds were then placed on each side of the dorsum of each mouse in the shaved area. For each animal, the four wound surface areas on a single side were averaged. Wound data reflect the mean of those averages with each animal’s average representing one point in the analysis.

Before the experiments reported, the wound measurement procedure was optimized and wound measurements were made on d 1, 4, and 7 after wounding. In the preliminary experiments, control mouse wounds healed 5% relative to baseline during the first day after wounding. Because wound surface areas were 95% of baseline, it was determined that the differences in healing on d 1 were too small to discern reproducibly. Many wounds were completely closed by d 7. On d 4, wounds were reliably open with reproducibly varying degrees of wound closure corresponding to the treatment group. Control mouse wound surface areas were approximately 60% of baseline. This was a large enough change to visualize reliably.

In a modification of previous protocols (10, 28), full thickness wounds were introduced with a small, dedicated scissors. Wounds were circular. Standardization was enhanced by keeping diameters perpendicular to the spine 10 mm. Full thickness wounds are those that include both the epidermis and that portion of the dermis that separates freely from scapular tissue. Wounds were placed either 2 mm to the right or 2 mm to the left of the midline. Wounds were left open to heal by secondary intention. Bleeding was minimal. Wounds were measured upon wounding to account for variability in two-dimensional size. Wounds were measured 4 d after injury.

Five-week-old CD-1 mice were randomized into four groups (Fig. 1). The first two groups had six mice each and were evaluated in a crossover study. Each mouse in the crossover study was evaluated twice: once after a 150 ng daily T₃ treatment and once after treatment with vehicle alone. Six mice received T₃ first followed by a 3-month wash-out/healing completion period. The animals were then evaluated after treatment with topical vehicle alone. Six mice started with vehicle alone and received the T₃ treatment after the 3-month wash-out/healing completion period. In each experiment, wounds on one side the dorsum received the assigned treatment and wounds on the other side were left untreated.

FIG. 1. Wound healing protocol. Matched mice were randomized into four groups. The first group had wound healing rate measured after treatment with vehicle alone. The second group had wound healing rate measured after daily treatment with 150 ng topical T₃. The third group had wound healing rate measured after daily treatment with 3.8 μg topical T₃. The fourth group had wound healing rate measured after daily treatment with 250 ng ip T₃. After a prolonged washout/healing period, the experiment was repeated with the first two groups reversed. The first group had the wound healing rate measured after daily treatment with 150 ng topical T₃ and the second group had the wound healing rate measured after treatment with vehicle alone.

High-dose topical T₃ treatment and ip T₃ treatment

The third group of mice was treated with a higher dose T₃ cream prepared such that each 30 μl application contained 3.8 μg of T₃. The fourth group was treated with supraphysiologic ip T₃ (0.23 μg ip T₃ daily). Neither of these groups was reevaluated in the crossover part of the study.

Relative wound surface area calculations

Upon wounding and on d 4 after wounding for each analysis, all animals were photographed with a digital Samsung (Ridgefield Park, NJ) Digimax130. All photographs included a standard ruler to maintain size consistency. All photographs were printed on 20-lb white paper stock with a brightness of 84. Wound photographs from each animal were carefully cut from the photographs and weighed using an Ohaus (Pine Brook, NJ) Analytical Plus scale. Thus, irregular borders could be accommodated in the calculations. Each animal served as its own control with percent wound closure representing the weight of wound photographs for a specific mouse on d 4 relative to the weight of wound photographs for the same mouse on the day of wounding. Surface areas were calculated from the wound picture weights relative to known standards.

Measurement of thyroid hormone levels

To ascertain thyroid hormone status, all mice were eye bled. Serum total thyroxine levels and serum total triiodothyronine levels were measured with standard RIA kits (ICN, Orangeburg, NY). Unlike other thyroid hormone kits that use antinouse antibodies, the ICN kits use antirabbit antibodies and avoid spuriously elevated readings in mice. Thyroid hormone levels for mice fall at the low end of the human range so the human standards included in the kit were used. T₃ dose-response curves were established for the different treatments. For the dose response curves, T₃ concentrations were determined before treatment and then on the first treatment day at the following times after treatment: 1, 2, 6, and 24 h.

K6 immunohistochemistry

Skin samples were taken from mice treated topically with supraphysiologic T₃ (3). In addition, skin samples were taken from surgically hypothyroid mice (see below). K6 protein expression was determined with immunohistochemistry as previously described (10). In brief, paraffin-embedded skin samples from tested mice were deparaffinized. Sections were incubated with unlabeled primary K6 antibody (Covance, Berkeley, CA). Visualization was achieved with a standard second antibody immunoperoxidase staining kit (Unitect ABC kit, Oncogene Research Products, Boston, MA). K6 protein expression data are presented as the percentage of hair follicles staining with K6 antibody.

Establishing mouse hypothyroidism

Fourteen age-, sex-, and size-matched CD-1 mice (Charles River) were thyroidectomized (by surgical thyroidectomy). To ascertain hypothyroidism, all mice were eye bled at wk 6 and serum total thyroxine levels measured with the standard RIA kit discussed above. As with all the murine studies, control mice were subject to the anesthesia, shaving, eye bleeding, and histological analysis used for the treatment animals.

Statistical analysis

For the wound healing study, statistical analysis was performed with Student’s t test. For the crossover part of the study, statistical analysis was performed with Student’s independent sample t test. For the K6 antibody study, statistical analysis was preformed with ANOVA. Post hoc analysis then used Student’s independent sample t test. Data are presented with SEM.

Results

Wound healing for control mice

For the control mice, full thickness dorsal skin wounds healed from a mean area of 79 ± 4 mm² to a mean area of 47 ±
4 mm² over the first 4 d after wound placement. Thus, on d 4 after wound placement, wounds were 59.6 ± 4% of their original areas. There was no difference in wound healing rate when mice were treated with vehicle alone versus when the wounds were left untreated. Also, there was no difference between healing rate in mice used as controls at the beginning of the experiment relative to mice used as controls 3 months later.

Wound healing for T₃-treated mice

For mice treated with 150 ng T₃ applied topically each day, wounds healed from a mean area of 79 ± 10 mm² to a mean area of 29 ± 8 mm² over the first 4 d after wound placement (Fig. 2, A and B). Thus, on d 4 after wound placement, wounds were 37.4 ± 6% of their original areas (P < 0.001 vs. control treatment; Fig. 2C).

As already stated in Materials and Methods, wounds were placed on both sides of the back of each mouse. Wounds on one side the dorsum received the assigned treatment and wounds on the other side were left untreated. For mice with T₃-treated wounds, the untreated wounds healed from a mean area of 79 ± 11 mm² to a mean area of 48 ± 9 mm² (39.7 ± 5%) over the 4 d after wounding. Thus, on d 4 after wound placement, wounds were 60.3 ± 5% of their original areas (P < 0.001 vs. T₃ treatment, P = 0.9 vs. control treatment).

Mice treated with 3.8 μg of topical T₃ daily did not have wound healing acceleration beyond that of the mice treated with 150 ng T₃ daily. Mice treated with IP T₃ did not have wound healing acceleration beyond that of control mice.

There was systemic absorption of topical T₃

Serum T₄ levels were measured in all mouse groups to ascertain the impact of T₃ treatment on the thyroid axis. Lower T₄ levels were interpreted to mean that there was systemic absorption of T₃ suppressing the endogenous axis. Control T₄ levels were 4.2 ± 0.4 μg/dl. T₄ levels in mice treated with 150 ng T₃ topically were 3.0 ± 0.9 (P = 0.3 vs. control). T₄ levels in 3.8 μg-treated mice were 1.8 ± 0.2 (P = 0.04), T₄ levels in the IP T₃-treated mice were 1.6 ± 0.4 (P = 0.01).

Serum T₃ curves were established for each treatment. Mice treated with IP T₃ had peak serum T₃ levels of 406 ± 27 ng/dl (P < 0.001). The peak occurred 1 h after injection. Two- and 6-h serum T₃ measurements suggested that the T₁/₂ was approximately 4 h. Twenty-four hours after injection, T₃ levels could not be discerned from those recorded at baseline.

Mice treated with topical T₃ did not have significant deviations from baseline with regard to their T₃ levels. The highest recorded T₃ level in the topically treated mice was in those mice treated with 3.8 μg of T₃. One hour after treatment, the 3.8 μg T₃ treatment mice had peak serum T₃ levels of 85 ± 15 ng/dl (P = 0.6 relative to control T₃ levels which were 70 ± 10 ng/dl).

Mice treated with supraphysiologic doses of T₃ had hair follicle K6 protein expression greater than control animals

When evaluated with immunohistochemistry, it was determined that application of supraphysiologic T₃ resulted in increased K6 protein expression (Fig. 3, A and B). The increased K6 protein expression was dose dependent (P < 0.001; Fig. 3C). Control mice had 43 ± 3% of hair follicles stain for K6. Mice treated with 3.8 μg topical T₃ daily had 98 ± 2% more hair follicles staining with K6 (85 ± 2%, P < 0.001 vs. control mice). Mice receiving 760 ng topical T₃ daily had 49 ± 5% more hair follicles stain with K6 than controls (64 ± 5%, P < 0.05 vs. control and P < 0.01 vs. 3.8 μg dose). Mice receiving 150 ng topical T₃ daily trended toward increased K6 staining (48 ± 5%), but the sample size was too small to confirm significance (P = 0.4). Epidermal keratinocytes did not stain consistently for K6. There was no detectable difference in epidermal K6 staining among treatment groups.

K6 protein expression was less in hypothyroid mice than in control mice

The K6 protein expression analysis was extended to evaluate samples taken from hypothyroid mice. Relative to baseline, T₄ levels in thyroidectomized mice were 84% lower (4.2 ± 0.4 μg/dl for euthyroid mice vs. 0.66 ± 0.5 μg/dl for hypothyroid animals, P < 0.001). In the samples from the hypothyroid mice, 23 ± 3% fewer follicles stained for K6 (P < 0.05) relative to the control mice (Fig. 3D).
Discussion

Although the physiologic role of thyroid hormone in skin is not well understood, mounting evidence suggests that T3 plays an important role in epidermal proliferation. In the current study, we determined that topical T3 dramatically accelerates wound healing in mice. This represents the first demonstration that topical thyroid hormone may prove a useful wound healing agent.

We have determined that hypothyroidism in mice retarded wound healing by 50% (10). The current study does not address the issue of treating hypothyroid mice with topical thyroid hormone to accelerate wound healing. Systemic factors may play a role in the delayed wound healing from hypothyroidism. Such factors may not be treated with topical thyroid hormone.

It is no surprise that topical thyroid hormone should have action not seen with systemic thyrotoxicosis. Thyroid hormone action on epidermis is fundamentally dependent on the relative intracellular concentration of the active thyroid hormone, T3. Epidermis does not have a blood supply and receives blood borne constituents via dermis. In normal physiology, thyroid hormone seen by epidermal keratinocytes must come via dermal fibroblasts. Thus, epidermal T3 is a product of the T3 made available systemically, T3 metabolism by the keratinocytes themselves, and the metabolism of T3 by adjacent dermal fibroblasts.

If T3 is delivered topically, a high T3 concentration can be achieved in keratinocytes absent the degree of thyrotoxicosis required with systemic administration. We speculate that there are antiproliferative factors stimulated by systemic thyrotoxicosis. The antiproliferative factors mitigate the degree of wound healing achieved when exogenous thyroid hormone is administered systemically. The small degree of improvement might result in some investigators failing to appreciate the change. This may explain why we were unable to achieve significant wound healing acceleration with ip T3.

There was some systemic absorption of topical T3. Although serum T3 concentrations were not altered in topically treated mice, serum T4 concentrations were diminished in mice receiving the higher doses of topical T3. It is unlikely that the T3 peak was missed in the topically treated animals when high T3 values could be measured in ip-treated animals for several hours after treatment. It is possible that the change in serum T3 levels for the topically treated mice was too small to be appreciated with the assay used. The T3 assay does not distinguish among low T3 levels well. The T4 assay is a more sensitive indicator of overall thyroid hormone status in this situation.

It is not clear whether the systemic absorption from the lower topical T3 treatment would be significant in larger studies. Although there was a downward trend in serum T4 levels in the mice receiving 150 ng T3 topically, the levels were not statistically different from control T4 levels. It is possible that a useful T3 dose could be delivered topically absent significant systemic thyroid hormone axis changes. Even with systemic absorption, topical T3 treatment could be useful for situations requiring rapid healing but with little concern for minor short-term perturbations to the thyroid hormone axis.

Studies suggest that the inactivating type 3 iodothyronine deiodinase has more activity in cutaneous tissues than the activating deiodinases types 1 and 2 (29, 30). Whereas topical T4 is inactivated by the skin, the current data suggest that supraphysiologic doses of topical T3 can substantially alter cutaneous physiology.

A major impediment to use of topical healing factors is the limiting cost associated. Current topical wound healing agents are expensive (31, 32). By contrast, thyroid hormone is inexpensive. Thyroid hormone acts in part to
stimulate epidermal growth factor expression (33). Although epidermal growth factor is expensive as an isolated wound healing agent, thyroid hormone might be an inexpensive means of achieving a similar result.

Previously, we demonstrated that proliferation-associated cytokeratin 6 gene expression is diminished in hypothyroidism and is dramatically stimulated with supraphysiologic doses of thyroid hormone (10). Although T3 stimulated K6 gene expression, to date only negative thyroid hormone response elements have been identified in the K6 promoter (24, 25). The mechanism of K6 stimulation by T3 must be indirect. In the current study, we extended our findings to protein expression. We demonstrated increased cutaneous K6 protein expression after topical T3 treatment.

Most K6 antibody staining occurred in the hair follicles not in the more superficial epidermal keratinocytes. Whereas T3 increased the percentage of hair follicle staining for K6 among tested groups, there was no discernable difference among more superficial epidermal samples. Hair follicle keratinocytes were the most prominent target for topical T3 apparent in our studies. Investigators have recognized a role for follicular keratinocytes in wound healing (26). Recent data suggest that hair follicular cells are integral to wound healing (27). It is possible that the T3 cream collected in the hair follicles for purely physical reasons. Even so, it would follow that T3 stimulation of follicular keratinocytes would accelerate wound healing.

Wound healing was accelerated using a dose of topical T3 that did not have significant effect on K6 protein expression above control levels. Although K6 may be necessary for optimal physiologic wound healing (10, 23), supraphysiologic topical T3 may accelerate wound healing further by a mechanism independent of additional K6 protein expression. Alternatively, immunohistochemistry may be limiting the interpretation. Per se, immunohistochemistry requires protein expression to meet a certain threshold for detection. It may be that 150 ng of daily topical T3 stimulated K6 protein expression above control but that the immunohistochemistry was not sufficiently sensitive to make the distinction.

The 3.8-μg T3 dose did not increase wound healing acceleration above that seen with the 150-ng T3 dose. We speculate that a lower dose of T3 could be administered and accelerate wound healing with even less systemic consequence than seen with the 150-ng T3 dose.

The current study did not evaluate wound quality. It is possible that healed wounds have larger or weaker scars, although we did not discern such a problem grossly. Were thyroid hormone accelerated scars to prove inferior, it might be speculated that the treatment would be useful for situations requiring rapid healing with little concern for the scar quality.

A unique aspect of skin is the possibility to target it directly via topical treatment. Thus, we can take advantage of thyroid hormone’s anabolic effect on skin achieved with topical administration. Our study is the first to demonstrate that topical T3 can accelerate wound healing in vivo. The data support our previous hypothesis that T3 is necessary for optimal wound healing. Now, we further propose that topical thyroid hormone may be an inexpensive agent to hasten healing of certain wounds.

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