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A Role for Thyroid Hormone in Wound Healing through Keratin Gene Expression

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The importance of thyroid hormone (TH) in wound healing is not well understood. To gain insight, we evaluated the impact of TH deficiency on wound-healing genes in cultured keratinocytes. By RT-PCR, keratin 6a (K6a) and 16 (K16) gene expression in TH-replete cells was 3.8- (P < 0.006) and 1.9-fold (P < 0.05) greater, respectively, than expression in TH-deficient cells. By real-time PCR, TH replete cell expression of K6a, K16, and K17 was greater than in deficient cells 18- (P < 0.001), 10- (P < 0.001), and 4-fold (P < 0.005), respectively. To examine TH requirement for optimal wound healing, we contrasted TH-deficient vs. ip T3-treated mice. Four days after wounding, ip T3-treated mice had twice the degree of wound closure as hypothyroid mice (P < 0.001). By RT-PCR, K6a and K17 gene expression from control mouse skin was greater than from hypothyroid mouse skin: 5- (P < 0.001) and 1.7-fold (P < 0.05), respectively. T3 is necessary for the keratinocyte proliferation required for optimal wound healing. T3 exerts influence by stimulating expression of the wound-healing keratin genes. Thus, for hypothyroid patients undergoing surgery that cannot be delayed until euthyroidism is achieved, our data support T3 treatment for the perioperative period. (Endocrinology 145: 2367-2361, 2004)

The importance of thyroid hormone (TH) to wound healing is not well understood. Lennox and Johnston (1) reported that exogenous T3 improved the rate of wound healing in rats as well as the strength of the scars. They further reported that wound-healing speed was diminished in hypothyroid rats. Mehregan and Zamick (2, 3) observed that additional TH stimulated the rate and quality of wound healing in euthyroid rats. Scars were smoother in animals receiving T3 in drinking water. Pirk et al. (4) noted no change in wound healing with euthyroid hamsters receiving ip T3. Cannon (5) reported that hypothyroidism did not diminish wound strength in pigs, and Ladenson et al. (6) found no wound-healing deficits in hypothyroid humans.

In vitro, keratinocyte proliferation is retarded in T3-deficient medium relative to T3 replete medium (7). In vivo, topical T3 stimulates epidermal proliferation (7, 8), and topical triac, the mild TH analog, thickens skin (9).

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 (10-12), including the following: keratins 1 (K1) and 10 (K10) are associated with epidermal differentiation; K6a, K16, and K17 are associated with epidermal proliferation and wound repair; and K5 and K14 are expressed in the basal skin layer (their expression decreased as the skin cells differentiate).

In K6a knockout mice (13), the absence of K6a resulted in diminished superficial wound healing, but no change in full thickness wound healing. When K16 was overexpressed in cultured human keratinocytes, proliferation was enhanced (14). However, human K16 overexpressed in mice resulted in delayed wound healing in the transgenic animals (15, 16). Ex vivo investigation suggested that the K16 overexpression inhibited keratinocyte migration. A K17 knockout mouse suffered alopecia and compensatory K16 expression (17). The alopecia was less in those animals with greater K16 expression.

Although TH stimulates expression of K6a both in vitro and in vivo (18), to date only negative TH response elements have been identified for the keratin genes associated with proliferation (19-22). The following investigation was undertaken to ascertain the need for TH in optimal wound healing and to clarify the effect of TH on the expression of wound healing-associated keratin genes.

Materials and Methods

T3 regulation of keratin genes in cultured human keratinocytes

Cultured human keratinocytes (HaCaTs) were grown and maintained as previously described (7). Cultures were incubated overnight in medium supplemented with or absent 0.1 nm T3 (Sigma, St. Louis, MO). Serum for the culture medium was stripped of TH with two overnight AG 1-X8 incubations (Bio-Rad, Hercules, CA) and one activated charcoal incubation (Sigma). T3 was then supplemented in the concentration indicated. RNA was extracted from the cell cultures using a standard RNA extraction protocol (TRIzol reagent, Life Technologies, Inc./Invitrogen, Grand Island, NY). The cDNA for RT-PCR was created using Superscript II RNase H Reverse Transcriptase (Life Technologies, Inc., Rockville, MD).

Complete or partial sequences of human K6a, K10, K14, K16, and K17 are posted in the nucleotide section of National Library of Medicine's web site (23-26). From those sequences, the following primers were designed for the RT-PCR: K6a forward—5'-GGTGCGCGCCAACAGK6a reverse—5'-AAGGAGGCAAAGTTGGTCTAGGTAATCGAGCTATTGAG, K10 forward—5'-AATGAAAAATGAGTGTTTTCCTGK10 reverse—5'-CAGGACGCTGCCCTGATK14 forward—5'-GAGTCTGGACCCAGCATTCAAK14 reverse—5'-GCTTCACGCAGATTCTCATK16 forward—5'-GAGTGCTGTCGAGCAGTATTCCTT, K16 reverse—5'-TCCAAACTGGACAATCCAGAATCGK17 forward—

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5'-AGG AGA TGA CCT TGC CAT CCT, and K17 reverse—5'-GCC TGA TTG GCA GCG TGG AGG A.
All expression data were quantitated relative to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin genes that were simultaneously measured using the same techniques. At a minimum, each experiment was repeated on three independent occasions.

Quantitative PCR
Quantitative (real time) PCR using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) was performed with the following parameters: PCR amplification of 200 ng of single-stranded cDNA template was carried out using SYBR Green Master Mix (Applied Biosystems). Primers described above for the RT-PCR were used for the real-time studies also. Quantification of PCR products was determined using ABI Prism Sequence Detection System software (Applied Biosystems) and controlled for total RNA. Fold change was determined by comparison of experimental data with standards of known copy number.

Establishing mouse hypothyroidism
All animal experimentation described was conducted in accord with accepted standards of humane animal care. Fourteen age-, sex-, and size-matched CD-1 mice (Charles River, Boston, MA) were thyroidectomized (by surgical thyroidectomy). To ascertain hypothyroidism, all mice were eye-bled at wk 6, and serum total T₄ levels measured with a standard RIA kit (ICN Biomedicals, Inc., Orangeburg, NY). Unlike other TH kits that use antimouse antibodies, the ICN kit uses antirabbit antibodies and avoids spuriously elevated readings in mice. TH levels for mice fall at the low end of the human range so the human standards included in the kit were used.

Wound-healing analysis
At wk 6, all 14 mice were anesthetized with 3 × 3-cm midline areas of their backs delineated and shaved. Four 10-mm diameter full thickness wounds were then placed on the dorsum of each mouse in the shaved area. Relative wound surface areas were compared 4 d after injury (analysis no. 1). For each animal, the four wound surface areas were averaged. Wound data reflect the mean of those averages with each animal's average representing one point in the analysis.
After a 2-wk healing/washout period, all animals received 1 wk of daily 0.25-μg ip T₃ injections, and wounding analysis was repeated (analysis no. 2). After a 3-wk healing/washout period allowing animals to become hypothyroid again, wounding analysis was repeated on all animals (analysis no. 3).

Relative wound surface area calculations
Upon wounding and on d 4 postwounding for each analysis, all animals were photographed. All photographs included a standard ruler to maintain size consistency. All photographs were printed on the same paper stock. Wound photographs from each animal were carefully cut from the photographs and weighed. 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.

In vivo gene expression analysis
Tissue samples for RNA analysis were taken when the animals were killed and maintained in the RNA protecting agent, RNA later (Ambion, Austin, TX). RNA was later extracted from the skin samples using a standard RNA extraction protocol (TRizol reagent). Then cDNA for RT-PCR was created using Superscript II Reverse H Reverse Transciptase.
Complete or partial sequences of murine K6a and K17 are posted in the nucleotide section of National Library of Medicine's web site (27). From those sequences, the following primers were designed for the RT-PCR: K6a forward—5'-GGT TCC TGC TTC CCT CAT CGA CAA, K6a reverse—5'-CTG CGG AGG TTC GCG ATG TA, K17 forward—5'-CTT

![Fig. 1. A.](https://example.com/fig1a.png) In HaCaTs evaluated with RT-PCR, GAPDH-corrected K6a and K17 expression levels in T₃-treated cells were 3.8- and 1.9-fold greater than levels seen in T₃-deficient cells, respectively. No change in K17 expression could be discerned by RT-PCR. B. Representative gels show bands for keratin RNA extracted from T₃-treated and T₃-deficient cells with corresponding GAPDH bands below. C. In keratinocytes evaluated with real-time PCR, total RNA corrected K6a, K16, and K17 expression levels in T₃-treated cells were 18-, 10-, and 4.4-fold greater than those of T₃-deficient cells, respectively. ***, P < 0.001; **, P < 0.005; *, P < 0.05.
CCG TAC CAA GTT TGA GAC, and K17 reverse—5'-CGG TTC TTC TCC GCC ATC TTC.

All expression data were quantitated relative to the GAPDH housekeeping gene, which was simultaneously measured using the same technique.

**Statistical analysis**

Statistical analysis was performed with Student’s t test and ANOVA. Data are presented with SEM.

**Results**

*K6a, K16, and K17 gene expression was decreased in T₃-deficient keratinocytes*

RNA was extracted from human keratinocytes cultured overnight in medium supplemented with or absent 0.1 nM T₃. Extracted RNA was subjected to RT-PCR with keratin gene expression quantitated relative to the housekeeping gene, GAPDH. In T₃-treated cells, K6a gene expression was 3.8 ± 0.6-fold greater than in T₃-deficient cells (Fig. 1A and B; P < 0.005), and K16 gene expression was 1.9 ± 0.3-fold greater (P < 0.05). We found no change in K10, K14, or K17 gene expression in the TH-treated cells relative to the deficient cells.

RNA extracted from HaCaTs treated as above was analyzed with real-time PCR. Data were corrected for total RNA loaded. In T₃-treated cells, K6a gene expression was 18 ± 0.8-fold greater than in T₃-deficient cells (Fig. 1C; P < 0.001), T₃-treated K16 gene expression was 10 ± 0.2-fold greater than the expression in T₃-deficient cells (P < 0.001), and T₃-treated K17 gene expression was 4.4 ± 0.6-fold greater than in deficient cells (P < 0.005). T₃-treated cells did not have

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**Fig. 2.** A, In hypothyroid mice, wound healing lagged significantly behind healing in T₃ replete animals. Four days after wounding, skin wounds in hypothyroid mice were 28% closed. In the same mice treated with ip T₃ for 1 wk, wounds closed 54% after 4 d. When the mice were permitted to become hypothyroid again, wounds closed 26% after 4 d. **, P < 0.005. B, Representative hypothyroid mouse with baseline wounds. C, The same hypothyroid mouse with wounds on d 4. D, Representative ip T₃-treated mouse with baseline wounds. E, The same ip T₃-treated mouse with wounds on d 4.
statistically significant changes in K10 or K14 gene expression.

**Hypothyroid mice had retarded wound healing**

To determine whether the in vitro T₃-mediated keratin gene expression pattern could be reproduced in vivo, and whether that pattern would be associated with significantly poorer wound healing, we contrasted the impact of surgical hypothyroidism on wound healing and proliferation-associated keratin gene expression. 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) at wk 6.

Fourteen thyroidectomized mice were evaluated. Ten-millimeter diameter dorsal skin wounds were established in all animals 6 wk after thyroidectomy. Relative wound surface areas were determined 4 d after injury (analysis no. 1). After a 2-wk healing/washout period, all animals received 1 wk of daily 0.25-µg ip T₃ injections, and wounding analysis was repeated (analysis no. 2). After a 3-wk healing/washout period allowing animals to become hypothyroid again, wounding analysis was repeated on all animals (analysis no. 3).

At d 4, percent wound closure for ip T₃-treated mice was 208% that of hypothyroid animals (Fig. 2). In the ip T₃-treated mice, wound surface areas were reduced 54 ± 2.8% at d 4. In analysis no. 1, hypothyroid mouse wounds were closed 26 ± 5.3% at d 4 (*P* = 0.001 vs. ip-treated animals) and in analysis no. 3, hypothyroid mouse wounds were closed 26 ± 5.6% (*P* = 0.004 vs. ip-treated animals).

**Proliferation-associated keratin gene expression was diminished in hypothyroid mice**

RNA was extracted from the mouse epidermis samples, and keratin gene expression was determined with RT-PCR relative to GAPDH housekeeping gene control (Fig. 3). In the samples from the control mice, K6a gene expression was 5-fold greater than expression from hypothyroid mice (*P* < 0.001), and K17 gene expression was 1.7-fold greater (*P* < 0.05).

**Discussion**

The reported importance of TH in wound healing is contradictory. Although some authors report improved rates and quality of wound healing in response to TH (2, 3, 28–31), others report no apparent TH-mediated changes in wound healing (4–6). Studies supporting a role for TH used humans, rats, and guinea pigs. Negative studies used humans, pigs, and hamsters. To extend insight into TH action on skin, we investigated the consequence of modest hypothyroidism on skin wound healing in mice.

Moderate hypothyroidism resulted in poorer wound healing. Our hypothyroid mice had T₄ concentrations 16% of control levels. Humans undergoing near-total thyroidectomy would be expected to suffer that degree of hypothyroidism 3 wk postoperatively (T₄ fragmentation in humans is 1 wk).

Previously, we demonstrated that TH can stimulate epidermal proliferation in mice and rats (7, 8). Consistent with that finding, we demonstrated that proliferation-associated cytotkeratin 6a expression is diminished in hypothyroidism and is dramatically stimulated with supraphysiologic doses of topical TH (18). However, because K6a only mediates superficial wound healing (13), TH regulation of K6a cannot be the sole mechanism for the wound healing effect seen in vivo. Furthermore, previous investigators have reported that proliferation-associated cytokeratin genes contain inhibitory TH response elements in their promoters, suggesting that their expression would be decreased in the presence of TH.

The genes most associated with epidermal proliferation are K6a, K16, and K17. Their regulation by TH seems likely to play a role in mediating TH influence over epidermal proliferation. Although TH stimulates expression of proliferation-associated keratin genes, to date only negative TH response elements have been identified for these genes (21, 22). It is not known whether the above reflects TH induction of indirect keratin gene-stimulating pathways or the existence of unidentified positive TH response elements for the keratin genes.

Refuting the suggestion that the keratin genes serve only...
as markers of proliferation and are not proliferation factors themselves, Wojcik et al. (13) reported a wound-healing defect in K6α knockout mice. Paramio et al. (14) reported increased proliferation of cultured keratinocytes transfected with K16. Thus, TH stimulation of K16 expression provides a mechanism through which epidermal proliferation may be stimulated.

We presented three analyses of gene transcription. In vivo RT-PCR studies provided the best measure but were subject to interanimal variability. In vitro RT-PCR studies provided more precise measurements but suffered from masking small differences in transcription. Real-time PCR analysis included data from each PCR cycle and provided the most precise report with the RT-PCR pictures serving as a visual complement to the real-time data.

TH-deficient mice have poorer wound healing than seen with individual keratin gene knockout mice. TH acts on all three wound healing-associated keratin genes along with direct or indirect TH action on other genes required for optimal keratinocyte proliferation. With multiple genes affected, compensatory gene expression is prevented.

We propose that T₃ is necessary for the keratinocyte proliferation required for optimal wound healing. T₃ exerts influence by stimulating expression of the proliferation cytokinets 6α, 16, and 17. Thus, surgical patients should have thyroid status ascertained. For hypothyroid patients undergoing surgery that cannot be delayed until euthyroidism is achieved, our data support T₃ treatment for the perioperative period.

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