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Effect of Transforming Growth Factor-β1 on Parathyroid Hormone-Related Protein Secretion and mRNA Expression by Normal Human Keratinocytes In Vitro

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Parathyroid hormone-related protein (PTHrP) is produced by a wide range of neoplastic and normal cells, including keratinocytes where it may regulate growth and differentiation. Transforming growth factor-β (TGF-β) is a growth factor produced by many cells, including keratinocytes where it regulates epidermal homeostasis. TGF-β has been reported to be cosecreted with PTHrP in some neoplasms and to stimulate PTHrP production by neoplastic keratinocytes. However, the effects of TGF-β on PTHrP production by normal keratinocytes are not well characterized. In this study, we investigated the effects of endogenous and exogenous TGF-β on PTHrP production by normal human foreskin keratinocytes. PTHrP secretion, mRNA expression, and mRNA transcription in vitro were determined by N-terminal radioimmunoassay, ribonuclease protection assay, and transient transfections. PTHrP production and secretion of latent TGF-β activity were greatest in proliferating keratinocytes prior to and at confluence of monolayer cultures. TGF-β1 increased PTHrP mRNA expression by normal keratinocytes in a dose-dependent manner with maximal stimulation at 6–12 h after treatment. In addition, keratinocytes treated with a monoclonal anti-TGF-β antibody expressed decreased levels of PTHrP mRNA. The increased levels of PTHrP mRNA following TGF-β1 treatment were owing, at least partly, to an increase in PTHrP mRNA stability. TGF-β1 failed to activate transcription of the luciferase reporter gene driven by either the human or mouse PTHrP promoters. In conclusion, TGF-β1 functions as a paracrine or autocrine regulator of PTHrP production in normal human keratinocytes, and this may play a role in the regulation of keratinocyte proliferation or differentiation.

Key Words: Transforming growth factor β; differentiation; hypercalcemia of malignancy; keratinocyte; parathyroid hormone-related protein.

Introduction

Parathyroid hormone-related protein (PTHrP) was originally identified as a humoral factor responsible for the pathogenesis of humoral hypercalcemia of malignancy *(1,2)*. In addition to its production by human and animal neoplasms, PTHrP has been subsequently identified in many normal tissues, where its exact function is poorly understood *(1,2)*. PTHrP is expressed in particularly high levels by normal keratinocytes, where it may be involved in the regulation of keratinocyte growth and differentiation *(3–6)*. PTHrP has been reported to inhibit keratinocyte proliferation in vitro *(4,7)*. In addition, hairless mice injected with PTHrP antagonists exhibited hair growth with increased keratinocyte proliferation *(7)*. A major role of PTHrP in the maintenance of epidermal homeostasis was demonstrated by several in vivo studies using transgenic mice. Transgenic mice overexpressing PTHrP in their basal keratinocytes had defects in hair follicle development, whereas PTHrP "knockout" mice developed surface hyperkeratosis and sebaceous gland hypoplasia *(1,8,9)*. PTHrP acts on keratinocytes by binding to and activating an uncharacterized receptor, which uses calcium ion as a second messenger *(10)*.

Transforming growth factors-β (TGF-β) are a family of multifunctional peptides that affect cell growth and differentiation, and that modulate extracellular matrix formation *(11)*. These growth factors are produced by many cell types, including keratinocytes. The three major members of this family (TGF-β1, 2, and 3) have similar qualitative biological effects on different cell types in vitro *(12)*. They generally suppress the proliferation of epithelial cells, and many

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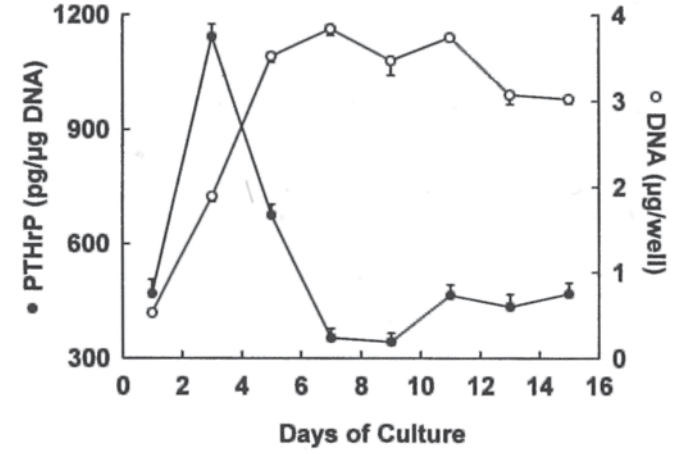

Fig. 1. Effect of cell density on PTHrP secretion by NHFK. Data represent mean ± SEM (n = 3/group).

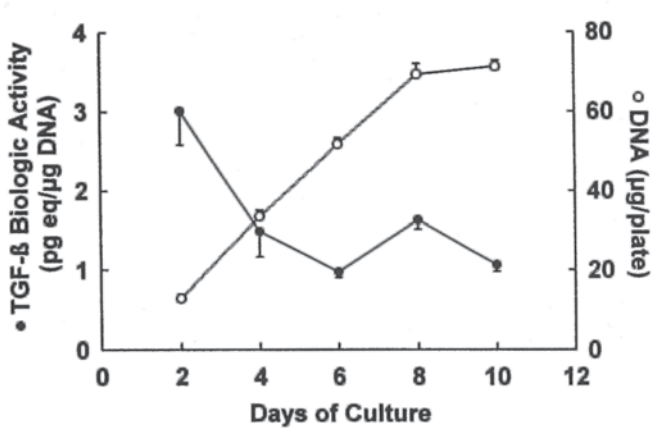

Fig. 2. Latent TGF-β production by NHFK. NHFK conditioned medium was acidified to activate latent TGF-β, and its biologic activity was measured by inhibition of [H^3]-uptake in mink lung epithelial cells (CCL-64). Data represent mean ± SEM (n = 3/group).

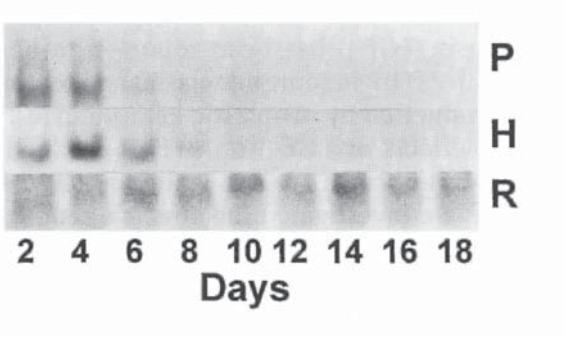

Fig. 3. Effect of cell proliferation on PTHrP, Histone 2B, and 18S ribosomal RNA expression in NHFK from d 1–14 in vitro, evaluated by Northern blot analysis. The cells were preconfluent on d 2, confluent on d 4, and postconfluent on d 6–18. P, PTHrP; H, Histone 2B; R, 18S rRNA loading control.

studies have shown that TGF-β inhibited keratinocyte proliferation in vitro *(13,14)*. The presumed mechanism involves the regulation of the retinoblastoma gene product (pRB) function by TGF-β *(13,15)*. In addition, although TGF-β clearly suppresses keratinocyte proliferation, it has also been reported to influence differentiation *(16–18)*. The major role played by TGF-β in the regulation of epidermal homeostasis has been underscored by the recent development of transgenic models. Overexpression of TGF-β in the epidermis results in a significant inhibition of epidermal development with inhibition of keratinocyte proliferation and abnormal differentiation *(19,20)*.

It has been reported that TGF-β is cosecreted with PTHrP in some neoplasms associated with humoral hypercalcemia of malignancy *(2,21,22)*. In addition, TGF-β has been shown to stimulate PTHrP secretion and mRNA expression by squamous cell carcinomas in vitro *(23)*. This suggests that TGF-β production by a neoplasm may play an important role in the induction of humoral hypercalcemia of malignancy in human patients or animals with specific tumors. TGF-β effects on PTHrP expression and secretion may not be unique to malignant epidermal cells, but this issue has not been investigated. The purpose of this study was to evaluate the effects of TGF-β on PTHrP production and mRNA expression by normal human foreskin keratinocytes (NHFK) in vitro in order to determine whether TGF-β functions as a regulatory cytokine of PTHrP production by normal keratinocytes.

Results

Effect of Cell Density on PTHrP and TGF-β Secretion

PTHrP secretion by NHFK was maximal when cells reached confluence (day 3 based on microscopic evaluation of the cultures) and decreased thereafter (Fig. 1). Active TGF-β was not detectable in the concentrated NHFK conditioned medium, as previously reported for other cell types *(24–26)*. However, TGF-β biological activity was detected after acid hydrolysis of the conditioned medium, indicating the presence of latent TGF-β *(24)*. Production of latent TGF-β by NHFK was greatest when the cultures were at low density and progressively decreased thereafter (Fig. 2). Preincubation of hydrolyzed conditioned medium with mouse monoclonal anti-TGF-β IgG_1 inhibited the TGF-β biological activity and confirmed the specificity of the assay to TGF-β (data not shown).

Effect of Cellular Confluence and Proliferation on PTHrP mRNA Expression

In order to determine the effects of cell density and proliferation on PTHrP mRNA expression, PTHrP mRNA expression by NHFK was measured in pre- and postconfluent keratinocytes. We have shown previously that NHFK maintain viability and protein production after differentiation in postconfluent monolayer cultures *(5)*. PTHrP mRNA expression was greatest in NHFK prior to cellular confluence and decreased after confluence (Fig. 3).

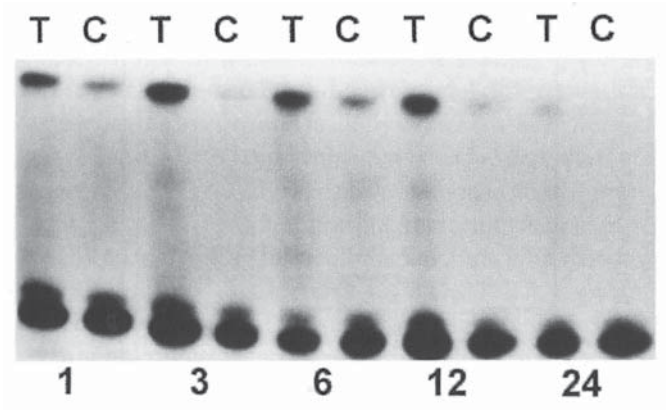

Fig. 4. Time-course of TGF-β1-mediated stimulation of PTHrP mRNA expression in NHFK evaluated by RNase protection assay. PTHrP mRNA expression was measured at 1, 3, 6, 12, and 24 h after addition of TGF-β1 (T) or vehicle control (C). Upper band, PTHrP; lower band, GAPDH, loading control.

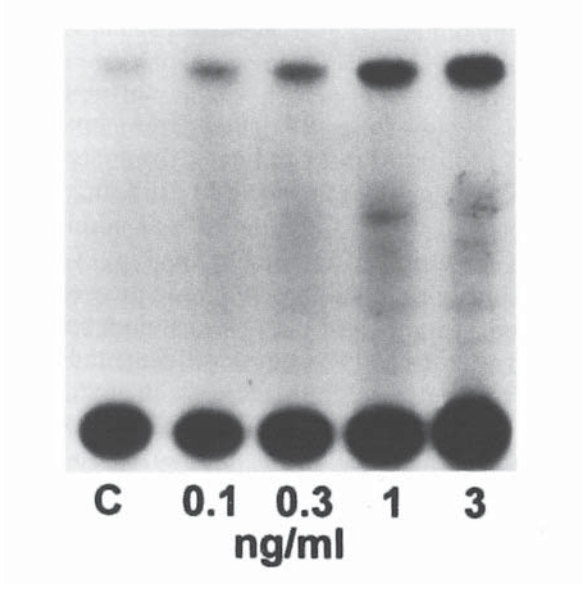

Fig. 5. Effect of TGF-β1 on PTHrP expression in NHFK, evaluated by RNase protection assay. Cells were treated with vehicle, 0.1, 0.3, 1.0, or 3.0 ng/mL TGF-β1 for 6 h. Upper band, PTHrP; lower band, GAPDH, loading control.

Histone H2B mRNA expression exhibited a similar pattern to PTHrP mRNA expression with greatest levels at cellular confluence and lower levels thereafter (Fig. 3).

Effect of TGF-β1 on PTHrP mRNA Expression and Stability

TGF-β1 treatment (3 ng/mL) resulted in a twofold increase in PTHrP mRNA expression by NHFK as early as 1 h after treatment compared to vehicle treatment (19.9 and 9.9 optical density units [ODU], respectively). The stimulatory effect of TGF-β1 was maximal after 3 h of treatment and sustained for up to 24 h. PTHrP mRNA expression was increased sevenfold between 3 and 6 h after TGF-β1 treatment (Fig. 4).

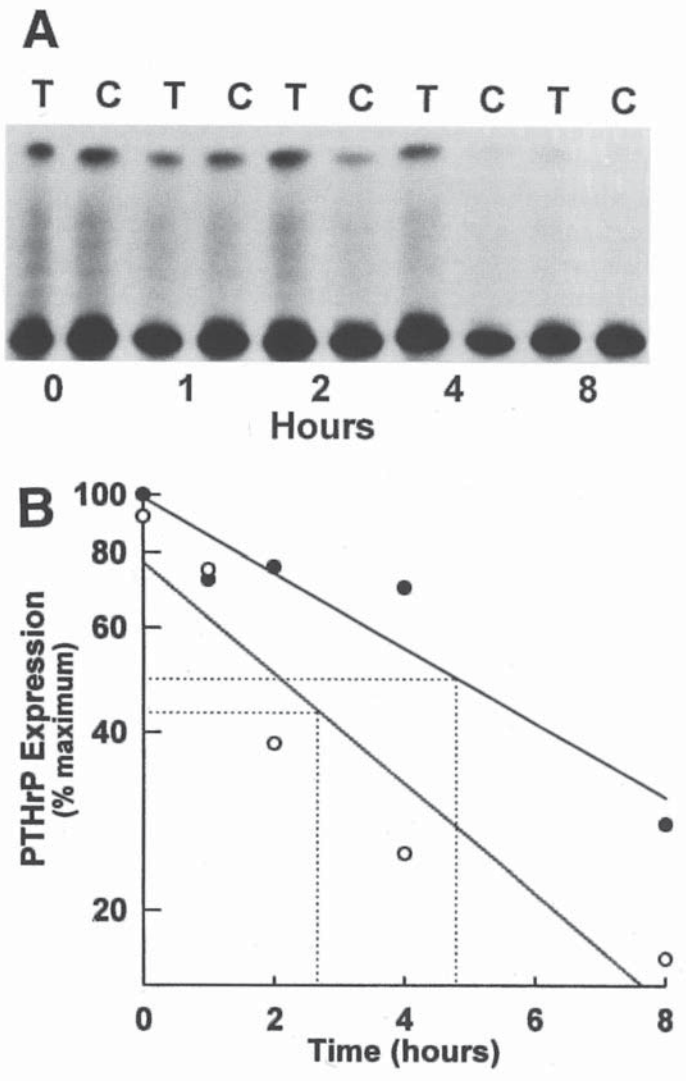

Fig. 6. (A) Effect of TGF-β1 on PTHrP mRNA stability in NHFK. TGF-β1 (T, 3 ng/mL) or vehicle control (C) was added to NHFK cultures treated with actinomycin-D, RNA was collected at 0, 1, 2, 4, and 8 h, and PTHrP mRNA expression was evaluated by RNase protection assay. Upper band, PTHrP; lower band, GAPDH, loading control. **(B)** Analysis of PTHrP mRNA half-life. The autoradiograph in Fig. 6A was scanned with a laser densitometer. PTHrP signal intensities were normalized to GAPDH in TGF-β1 (3 ng/mL) or vehicle-treated NHFK cultures and plotted against time on a log-linear scale. Maximum PTHrP mRNA expression (TGF-β1 at T_0) was assigned a value of 1.0. The half-life of PTHrP mRNA was estimated by the slope of the lines.

TGF-β1 increased PTHrP mRNA expression in a dose-dependent manner at all concentrations examined (0.1, 0.3, 1.0, and 3.0 ng/mL). Optical density measurements of laser-scanned autoradiograms revealed a three- to sevenfold increase in PTHrP mRNA levels compared to vehicle treatment (Fig. 5). TGF-β1 increased PTHrP mRNA stability in NHFK compared to vehicle treatment. PTHrP mRNA half-life was approx 4.8 h in TGF-β1-treated NHFK compared to 2.7 h in vehicle-treated NHFK (Fig. 6A,B). The addition of anti-TGF-β antibody decreased PTHrP

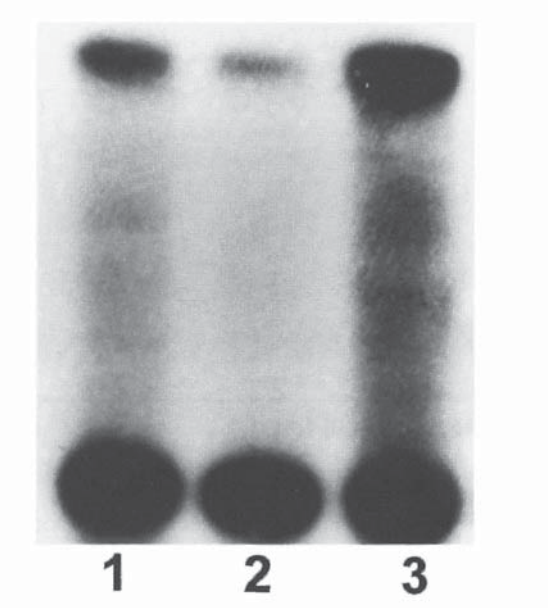

Fig. 7. Effect of neutralizing anti-TGF-β MAb on basal PTHrP expression. NHFK cultures in basal medium were treated with either 2 μg/mL mouse IgG control antibody (lane 1), 2 μg/mL mouse monoclonal anti-TGF-β antibody (lane 2), or 3.0 ng/mL TGF-β1 (lane 3) for 6 h, and PTHrP mRNA was measured by RNase protection assay. Upper band, PTHrP; lower band, GAPDH, loading control.

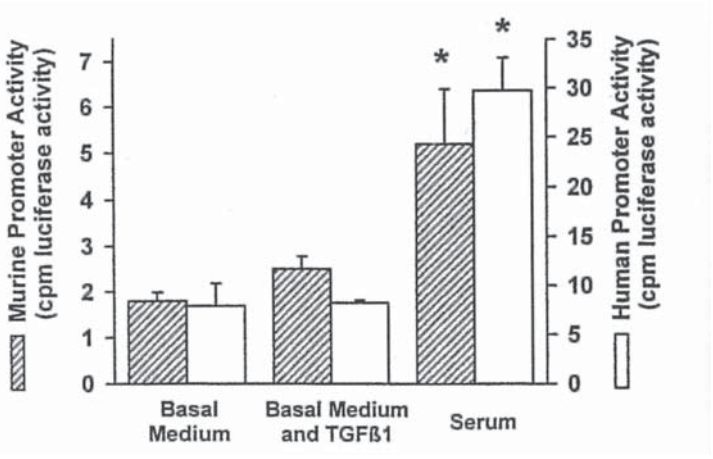

Fig. 8. Effect of TGF-β1 on PTHrP gene expression. NHFK were transiently transfected with constructs containing the 5'-flanking DNA of either the human or mouse PTHrP genes ligated to a luciferase vector. After transfection, NHFK were treated for 24 h with basal medium or basal medium supplemented with TGF-β1 or serum. Serum treatment resulted in a significant increase in luciferase activity (positive control). There was no increase in luciferase activity following TGF-β1 treatment.

mRNA levels in NHFK approx 50% compared to control IgG (Fig. 7).

Effect of TGF-β1 on PTHrP Promoter Activity

Both the mouse and human PTHrP promoters were significantly ($p < 0.01$) upregulated two- and threefold respectively, by serum treatment for 24 h, which served as the positive control. TGF-β1 did not significantly affect expression of either the human or mouse PTHrP promoter/luciferase reporter genes (Fig. 8).

Discussion

These studies demonstrated that PTHrP expression and secretion by NHFK is strongly correlated with keratinocyte growth and differentiation and that TGF-β is a positive regulator of PTHrP mRNA expression by NHFK in vitro. In addition, the TGF-β-mediated increase in PTHrP expression by NHFK is owing, at least partly, to an increase in the stability of PTHrP mRNA.

The recent development of transgenic models for PTHrP has shown that PTHrP is an important regulator of epidermal homeostasis *(8,9)*. In vitro data also support a major role for PTHrP in the regulation of keratinocyte growth and differentiation *(4,5,27)*. In this study, we demonstrated that secretion of immunoreactive PTHrP by NHFK was maximal when cells reached confluence in vitro and decreased thereafter, despite continued metabolic activity of the cells. This confirmed data previously reported by our laboratory *(5,28)*. The decrease in PTHrP secretion by NHFK after confluence correlated with decreased steady-state PTHrP mRNA levels and decreased proliferation as indicated by histone H2B mRNA expression. Confluence of NHFK in vitro has been associated with differentiation and mimics some aspects of epidermal differentiation in vivo *(29,30)*. We have previously reported that the production of PTHrP by keratinocytes cultured with low calcium conditions decreased after the cells became confluent, and that this decrease correlated with an increase in involucrin (a marker of keratinocyte differentiation) content of the cultures *(5)*. In addition, we have reported that normal human keratinocytes grown on collagen type I differentiated more rapidly, had a lower proliferation rate, and produced lower levels of PTHrP *(28)*. The peak level of PTHrP expression at confluence observed in the present study, as well as our observations in other investigations, suggest that PTHrP may be involved in a local control mechanism promoting NHFK growth arrest and differentiation in vitro.

A role for PTHrP in the differentiation of keratinocytes in vitro has been suggested by several investigators. Our laboratory has reported that increased differentiation of normal keratinocytes was associated with a decrease in PTHrP expression and secretion in vitro *(5,28)*. Loewik et al. observed that induction of differentiation in both normal and malignant keratinocytes was associated with the inhibition of PTHrP production *(6)*. In addition, stable transfection of a keratinocyte cell line with an antisense PTHrP expression construct resulted in increased proliferation and decreased differentiation of the cells *(4,27)*. These data suggest that PTHrP directly or indirectly stimulates differentiation of keratinocytes in vitro.

Calcium has potent prodifferentiation effects on keratinocytes *(31,32)*. Increased intracellular calcium concentration has been reported to induce terminal differentiation of keratinocytes in vitro and is likely central to the differentiation process of the epidermis in vivo *(33–36)*. Orloff et al. demonstrated a highly sensitive response to N-terminal PTHrP in neoplastic and normal keratinocytes characterized by an increase in intracellular calcium, which does not involve the well-characterized PTH/PTHrP G-protein-linked receptor *(10)*. This suggests that keratinocytes and squamous carcinoma cells possess high-affinity receptors specific for N-terminal PTHrP and that may regulate intracellular calcium transport. Therefore, PTHrP may stimulate keratinocyte differentiation through mobilization of intracellular calcium.

TGF-β is produced predominantly by cells of the basal epidermis, and has been shown to inhibit proliferation and induce reversible differentiation of keratinocytes in vitro *(17,37,38)*. The antiproliferative and prodifferentiating effects of TGF-β1 on keratinocytes in vitro appear to involve separate pathways. TGF-β1 inhibits epithelial cell growth mostly by inducing a G1 cell-cycle arrest that is independent of extracellular calcium *(39,40)*. In contrast, TGF-β1-induced terminal differentiation of keratinocytes in vitro is indirect and requires high extracellular calcium conditions (1.8 m*M*) *(39)*. Since exogenous and endogenous TGF-β1 appeared to be a potent stimulator of PTHrP mRNA expression by NHFK, we suggest that TGF-β1 induction of terminal differentiation may involve PTHrP. Holick et al. have shown evidence that the prodifferentiation and antiproliferative activities of 1,25 dihydroxycholecalciferol on keratinocytes may be mediated by PTHrP *(41)*. A similar mechanism also may exist for TGF-β.

Malignancies of keratinocyte origin can be complicated by the syndrome of humoral hypercalcemia of malignancy *(42,43)*. It has been shown that the ability of a squamous call carcinoma to be associated with humoral hypercalcemia of malignancy is ultimately a function of its level of PTHrP gene expression *(44)*. In addition, several squamous carcinoma cell (SCC) lines produce high PTHrP levels *(6)*. These data do not support a role for PTHrP as a prodifferentiation factor, since many SCC lines are characterized by a poorly differentiated phenotype *(6)*. However, most SCC lines are also characterized by defects in their terminal differentiation program and altered cellular response to various cytokines *(6,32,45,46)*. Therefore, it is likely that these SCC lines do not respond to PTHrP in a physiologic way.

The rapid effect of TGF-β1 on PTHrP mRNA levels suggested a direct effect of TGF-β1 at the level of gene transcription or mRNA stability. TGF-β1 has been previously reported to stabilize PTHrP mRNA in an epidermal squamous cancer cell line (COLO16) *(22)*. This is similar to our findings in NHFK. However, in COLO16 cells, TGF-β1 also stimulated PTHrP transcriptional activity *(22)*. Using transient transfection experiments, we did not demonstrate significant effects of TGF-β on PTHrP gene transcription. Serum is known to be a potent stimulator of PTHrP expression in keratinocytes *(47)*. Therefore, we used serum as a positive control in our transfection experiments and showed a significant increase in PTHrP gene transcription following serum treatment. It is possible that the lack of response to TGF-β observed in the transfection experiments was owing to the absence of critical regulatory elements in the constructs used. However, neoplastic keratinocytes have been shown to have altered cellular responses to various cytokines, including TGF-β1 *(45,46)*. In addition, PTHrP expression and secretion by human keratinocytes has been shown to be dysregulated during neoplastic transformation *(47)*. Therefore, our results may reflect differences in TGF-β effects on PTHrP gene transcription between normal and neoplastic keratinocytes.

In these studies, we also have demonstrated that endogenously produced TGF-β is a potential autocrine or paracrine factor involved in the expression of PTHrP mRNA by NHFK, since treatment with anti-TGF-β antibodies decreased PTHrP mRNA levels. Anti-TGF-β antibody treatment has previously been reported to inhibit PTHrP secretion in canine and human SCC lines *(22,23)*. Our results demonstrate that a similar mechanism is also functional in normal keratinocytes.

In summary, TGF-β1 increased PTHrP mRNA expression and stability by NHFK in vitro. TGF-β-mediated stimulation of PTHrP expression in normal keratinocytes may be involved in the paracrine control of keratinocyte proliferation, growth arrest, or terminal differentiation. Further investigations will be necessary to elucidate the mechanism by which the expression and interaction of TGF-β and PTHrP in normal keratinocytes regulate normal epidermal homeostasis.

Materials and Methods

Keratinocyte Cell Culture

Serum-free culture of NHFK was conducted as previously described by Boyce and Ham *(48)*. Briefly, neonatal foreskin tissues were minced and incubated for 48 h in 0.2% trypsin (type III, Sigma, St. Louis, MO) at 4°C. The epidermis was removed from the underlying dermis and dispersed in MCDB 153 (Sigma) medium containing 10% fetal bovine serum (Gibco BRL, Grand Island, NY) in order to inactivate the trypsin. The cell suspension was centrifuged, and the pellet was resuspended in keratinocyte serum-free medium (SFM) (Gibco-BRL) containing low calcium (0.08 m*M*), epidermal growth factor (EGF, 5 ng/mL), and bovine pituitary extract (BPE, 35 μL/mL). Keratinocytes were placed in 75-cm^2 tissue-culture flasks, and incubated at 37°C in 95% air/5% CO_2. The medium was replaced in stock cultures every 48 h and cells passaged at a ratio 1:10 at 70% confluence. NHFK at passages 2–5 were used for the studies.

Collection of Keratinocyte Conditioned Medium

To determine PTHrP and TGF-β content in NHFK conditioned medium, cultures of NHFK were established at 30% confluence in 12-well tissue-culture plates (Costar, Cambridge, MA) and maintained for 15 d. Conditioned medium was collected from triplicate wells 24 h after each medium change.

DNA Quantification

Following collection of conditioned medium, cells in 12-well plates were lysed in 1 mL/well GITC (4 *M* guanidinium isothiocyanate, 25 m*M* sodium citrate, 0.5% sarcosyl) as previously described *(28)*. DNA content of cell lysates was determined by DNA fluorimetry using a TKO fluorimeter and Hoechst 33258 dye (Hoefer Scientific Instruments, San Francisco, CA). Calf thymus DNA (100 µg/mL) served as a calibration control. DNA content of cell lysates was used to normalize PTHrP and TGF-β production by NHFK.

PTHrP Nonequilibrium Radioimmunoassay (RIA)

The RIA for PTHrP was performed as described by our laboratory using chicken polyclonal anti-PTHrP-(1-36) antibody *(49)*. [Tyr^{36}]-PTHrP-(1-36) (Bachem, Torrance, CA) was radioiodinated with ^{125}I and Iodogen (Pierce, Rockford, IL) and purified using reverse-phase HPLC. Polyclonal chicken anti- PTHrP-(1-36) antibodies (10 mg/mL in PBS, 1:1000 dilution) were added to borosilicate tubes containing standards or medium samples (100 µL) and assay buffer (20 m*M* sodium phosphate, pH 7.4, 140 m*M* NaCl, 50 m*M* EDTA, 0.5% BSA, 0.1 sodium azide), and were incubated for 48 h at 4°C. Iodinated PTHrP (1-36) was added (10,000 cpm/tube) and dextran-coated charcoal (5 mg/mL activated charcoal, 0.5 mg/mL dextran) was utilized to separate the bound from free PTHrP after 48 h. The supernatant was measured in a γ-radiation counter and PTHrP content quantified by log-logit transformation using Securia 1.0 (Packard Instruments, Downers Grove, IL). The RIA was sensitive to 0.1 ng PTHrP (1-36)/mL of medium.

Thymidine Incorporation Inhibition Assay for TGF-β Biological Activity

Mink lung epithelial cells (CCL-64 cells, American Type Culture Collection, Rockville, MD) were used to detect TGF-β activity in NHFK conditioned medium. Active TGF-β inhibits thymidine incorporation in the CCL-64 cells *(50)*. NHFK were grown in 90-mm plates with keratinocyte-SFM. Conditioned medium was collected after 48 h and concentrated 10-fold using sterile 10,000 mol-wt cutoff centrifugal concentrators (Centricon, Amicon Inc., Beverly, MA). Concentrated conditioned medium was divided in half. One-half was left at neutral pH and used to determine the biologically active fraction of TGF-β. The second half was acidified to pH 1.0 with 1 *N* HCl in order to activate latent TGF-β present in the CM. Acidified CM was neutralized with 1 *N* NaOH. CCL-64 cells (50,000 cells/well) were added to 48-well tissue-culture plates in DMEM-F12 medium containing 10% FBS. Human recombinant TGF-β1 (R&D Systems) was used for generation of a standard curve. Test and control substances were added, and the plates were incubated for 36 h at 37°C. [^{3}H]-thymidine (1 µCi/mL) was added to the cultures, and the cells were incubated for an additional 12 h at 37°C. Cells were washed with Hank's Balanced Salt Solution (HBSS) and ice-cold 10% trichloroacetic acid (TCA). Acid-insoluble precipitate was dissolved in 0.5 *N* NaOH and quantified in a liquid scintillation spectrometer. The amount of TGF-β biologic activity in NHFK CM was calculated by log-logit transformation and plotted against the standard curve. To demonstrate the specificity of the assay, hydrolyzed conditioned medium was preincubated for 2 h at room temperature with 2 µg/mL mouse monoclonal anti-TGF-β IgG_1 (#2G7.5A9, generously provided by Genentech Inc., South San Francisco, CA), which neutralizes TGF-β1, 2, and 3.

TGF-β1 Treatment of NHFK

In order to determine the effect of TGF-β on PTHrP mRNA expression by NHFK, NHFK were grown to 70% confluence in 90-mm plates and then maintained overnight in keratinocyte SFM not supplemented with EGF and BPE (basal medium) to delay growth. Fresh medium containing TGF-β1 (R&D Systems, Minneapolis, MN) or vehicle (0.1% bovine serum albumin in 4 m*M* HCl) was then added to the cultures. To determine the time-course of TGF-β1 effect on PTHrP mRNA expression, TGF-β1 (3 ng/mL) or vehicle was added to the medium, and total RNA collected after 1, 3, 6, 12, and 24 h. To evaluate the dose–response relationship of TGF-β1 effect on PTHrP mRNA expression, TGF-β1 (0.1, 0.3, 1.0, and 3.0 ng/mL) or vehicle was added to the existing culture medium, and RNA was collected after 6 h of treatment.

Anti-TGF-β Antibody Treatment

To determine the effect of endogenously produced TGF-β on basal PTHrP mRNA expression, 2 µg/mL of neutralizing monoclonal anti-TGF-β antibody (#2G7.5A9, Genentech) or mouse IgG (control) were added to NHFK (70% confluent) in basal medium. RNA was collected 6 h after antibody treatment.

Collection of NHFK Total RNA

NHFK cultures were released from 90-mm plates by incubation in 1 mL trypsin-EDTA (0.05% trypsin, 0.53 m*M* EDTA) at 37°C for 20 min. Trypsin was neutralized with 250 µL HEPES buffer containing 1 mg/mL soybean trypsin inhibitor (Sigma). Cell suspensions were centrifuged and pellets lysed in 500 µL GITC and 4 µL β-mercaptoethanol. RNA was purified by two serial extractions using acid phenol (pH 4.0) and chloroform isoamyl alcohol (24:1) according to the method of Chomczynski and Sacchi *(51)*. Alternatively, RNA was collected directly from the 90-mm plates using RNazol B according to the instructions of the manufacturer (Gibco BRL). RNA was quantified by deter-

mination of UV absorbance at 260 and 280 nm using a GeneQuant UV spectrophotometer (Pharmacia Biotech, Piscataway, NJ).

Complementary RNA Probe Generation and Ribonuclease Protection Assay (RPA)

An α-[^{32}P]-UTP-labeled cRNA probe complementary to a 550 nucleotide coding region of human PTHrP in pSP65 *(51)* (generously provided by Genentech) was generated by in vitro transcription from the SP6 promoter using the Maxiscript kit (Ambion, Austin, TX). A 350 nucleotide cRNA probe complementary to human glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) was transcribed from a cDNA template (pTRI-GAPDH, Ambion) and used as a loading control. The probes were purified from the reaction mixture by 5% acrylamide/8 *M* urea gel electrophoresis and elution of the excised band overnight at 37°C. The resulting probes had a specific activity of approx 10^9 cpm/μg RNA.

PTHrP mRNA levels were evaluated using total RNA isolated from NHFK using the RPA II Kit (Ambion). Briefly, total RNA (20 μg) was coprecipitated with PTHrP and GAPDH cRNA probes (70,000 and 10,000 cpm, respectively). The hybridized PTHrP mRNA/cRNA probes were separated by 5% acrylamide/8 *M* urea gel electrophoresis. The gels were exposed to X-ray film (X-OMAT AR, Kodak, Rochester, NY) overnight at –80°C. PTHrP signal intensity was quantified using a laser densitometer (ImageQuant Series 300, Molecular Dynamics, Sunnyvale, CA) and was normalized to GAPDH signal intensity.

Northern Blot Assay for Histone H2B and PTHrP

PTHrP mRNA expression by NHFK was measured in pre- and postconfluent keratinocytes in order to determine the effects of cell density and proliferation on PTHrP mRNA expression. Keratinocytes were plated in 90-mm plates, and total RNA was collected at preconfluent, confluent, and postconfluent time-points. Total RNA was collected 4 h after addition of fresh medium.

Northern blot analysis was performed as described *(52)*. Briefly, 20 μg of RNA were loaded in each lane of a 1.2% agarose-formaldehyde gel and separated for 3 h at 100 V. The RNA was transferred to Duralon UV membranes (Stratagene Cloning Systems, La Jolla, CA) using the Turboblotter Rapid Downward Transfer System (Schleicher & Schuell, Keene, NH) and crosslinked using a UV Stratalinker 1800 (Stratagene). The membranes were hybridized to Histone H2B cDNA (Oncor, Gaithersburg, MD), a marker for S phase of the cell cycle, to evaluate the level of cellular proliferation in NHFK. An 111-bp cDNA probe for the coding region of PTHrP was generated by polymerase chain reaction (primers 5'-ACCAGCTCC TCCATGACA-3' and 5'-ATCAGAGCTACCTCGGAG-3') and used to evaluate the levels of PTHrP mRNA. The blots were also hybridized to a cDNA probe for ribosomal 18S RNA (Ambion) to serve as a loading control. The membranes were exposed to a phosphoimager (445 SI, Molecular Dynamics) for quantification and to Kodak X-OMAT AR film for 36 h for autoradiography.

Evaluation of PTHrP mRNA Stability

In order to determine the effect of TGF-β on PTHrP mRNA stability in NHFK, actinomycin D (5 μg/mL), an inhibitor of RNA transcription, was added to the basal medium containing either TGF-β1 (3.0 ng/mL) or vehicle. RNA was harvested from TGF-β1-treated and control NHFK cultures at 0, 1, 2, 4, and 8 h after treatment. PTHrP mRNA levels were plotted vs time based on ODU measurements of the laser-scanned autoradiogram in treatment and control groups. PTHrP half-life was estimated from the slope of the lines generated by these plots.

Transient Transfection of the PTHrP Promoter/Luciferase Constructs

To examine the effects of TGF-β1 on PTHrP gene expression, NHFK were transfected with constructs containing the 5'-flanking DNA of either the human or the mouse PTHrP genes ligated to a promoterless bacterial luciferase vector (pGL2-Basic, Promega, Madison, WI). The human PTHrP construct consisted of 950 bp of 5'-flanking DNA and included the untranslated exons III, IV, and the 5'-end of exon V, 20 bp short of the ATG translation start site. The construct contained the P2 and P3 PTHrP promoters and was generously provided by Larry Suva (Beth Israel Hospital, Boston, MA) *(53)*. The mouse construct consisted of 1.6 kb of the 5'-flanking DNA including the untranslated exons I, II, and the 5'-end of exon III. The construct contained DNA homologous regions to the human P2 and P3 PTHrP promoters *(54)*. The construct was generated by digestion of a 90-kb mouse genomic clone (Genome Systems, Inc., St. Louis, MO) containing the PTHrP gene with *Sst*I, end-filled with T7 DNA polymerase, ligated with the Marathon adapter (Clontech, Palo Alto, CA), PCR-amplified with the sense Marathon primer and a gene-specific antisense primer (5'-CTGGCTCTG GGGACCTGCAA-3') *(54)* in the 5'-region of exon 3 starting 19 bp upstream from the ATG start site, and cloned into pCRII (Invitrogen, San Diego, CA).

Transfection of normal keratinocytes was conducted in 12-well culture plates at 60% confluence using lipofectin (5 μL/well, Gibco BRL), which has been shown to be an efficient method for transfection of human keratinocytes *(55)*. Forty-eight hours after transfection, the cells were treated with either basal medium or basal medium supplemented with fetal bovine serum or TGF-β1. To standardize the transfection efficiency of each sample, cells were cotransfected with the CMV-β-galactosidase plasmid (Clontech), containing the β-galactosidase gene and cytomegalovirus (CMV) promoter. β-Galactosidase activity was assayed in cell extracts using the β-Galactosidase

Enzyme Assay System (Promega). Cell extracts were assayed for luciferase using the Luciferase Assay System (Promega) and a liquid scintillation counter (Packard Tricarb 2000CA, Downers Grove, IL) from triplicate wells. The luciferase values were standardized to β-galactosidase activity before statistical analysis.

Statistical Analysis

Data from PTHrP and TGF-β secretion studies and transient transfections were expressed as mean ± SE ($n = 3$) and analyzed by one-way analysis of variance using Instat version 2.0 (GraphPAD Software, San Diego, CA). Data were expressed as either pg PTHrP/mL conditioned medium or pg PTHrP/μg DNA. All experiments were repeated a minimum of three times with normal keratinocytes collected from different foreskin donors.

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References

1. Philbrick, W. M., Wysolmerski, J. J., Galbraith, S., Holt, E., Orloff, J., Yang, K. H., et al. (1996). *Physiol Rev*. **76,** 127–173.
2. Rosol, T. J. and Capen, C. C. (1992). *Lab Invest*. **67,** 680–702.
3. Atillasoy, E. J., Burtis, W. J., and Milstone, L. M. (1991). *J. Invest. Dermatol*. **96,** 277–280.
4. Kaiser, S. M., Sebag, M., Rhim, J. S., Kremer, R., and Goltzman, D. (1994). *Mol. Endocrinol*. **8,** 139–147.
5. Werkmeister, J. R., Merryman, J. I., McCauley, L. K., Horton, J. E., Capen, C. C., and Rosol, T. J. (1993). *Exp. Cell. Res*. **208,** 68–74.
6. Loewik, C. W., Hoekman, K., Offringa, R., Groot, C. G., Hendy, G. N., Papapoulos, S. E., et al. (1992). *J. Invest. Dermatol*. **98,** 198–203.
7. Holick, M. F., Ray, S., Chen, T. C., Tian, X., and Persons, K. S. (1994). *Proc. Natl. Acad. Sci. USA* **91,** 8014–8016.
8. Wysolmerski, J. J., Philbrick, W. M., Zhou, J., Broadus, A. E., and Milstone, L. M. (1994). *Proc. Natl. Acad. Sci. USA* **91,** 1133–1137.
9. Foley, J., Wysolmerski, J. J., Longley, J. B., Dreyer, B. E., Broadus, A. E., and Philbrick, W. M. (1997). *J. Bone Miner. Res*. **12 (Suppl. 1),** S163.
10. Orloff, J., Ganz, M. B., Ribaudo, A. E., Burtis, W. J., Reiss, M., Milstone, L. M., et al. (1992). *Am. J. Physiol*. **262,** E599–E607.
11. Lawrence, D. A. (1995). *Kidney Int*. **47 (Suppl. 49),** S19–S23.
12. Graycar, J. L., Miller, D. A., Arrick, B. A., Lyons, R. M., Moses, H. L., and Derynck, R. (1989). *Mol. Endocrinol*. **3,** 1977–1986.
13. Pietenpol, J. A., Holt, J. T., Stein, R. W., and Moses, H. L. (1990). *Proc. Natl. Acad. Sci. USA* **87,** 3758–3762.
14. van Ruissen, F., van Erp, P. E. J., de Jongh, G. J., Boezeman, J. B. M., van de Kerkhof, P. C. M., and Schalkwijk, J. (1994). *J. Cell Sci*. **107,** 2219–2228.
15. Pietenpol, J. A., Stein, R. W., Moran, E., Yaciuk, P., Schlegel, R., Lyons, R. M., et al. (1990). *Cell* **61,** 777–785.
16. Coffey, R. J., Jr., Sipes, N. J., Bascom, C. C., Graves-Deal, R., Pennington, C. Y., Weissman, B. E., et al. (1988). *Cancer Res*. **48,** 1596–1602.
17. Reiss, M. and Sartorelli, A. C. (1987). *Cancer Res*. **47,** 6705–6709.
18. Shipley, G. D., Pittelkow, M. R., Wille, J. J., Jr., Scott, R. E., and Moses, H. L. (1986). *Cancer Res*. **46,** 2068–2071.
19. Sellheyer, K., Bickenbach, J. R., Rothnagel, J. A., Bundman, D., Longley, M. A., Krieg, T., et al. (1993). *Proc. Natl. Acad. Sci. USA* **90,** 5237–5241.
20. Cui, W., Fowlis, D. J., Cousins, F. M., Duffie, E., Bryson, S., Balmain, A., et al. (1995). *Gene Dev*. **9,** 945–955.
21. Insogna, K. L., Weir, E. C., Wu, T. L., Stewart, A. F., Broadus, A. E., Burtis, W. J., et al. (1987). *Endocrinology* **120,** 2183–2185.
22. Kiriyama, T., Gillespie, M. T., Glatz, J. A., Fukumoto, S., Moseley, J. M., and Martin, T. J. (1993). *Mol. Cell. Endocrinol*. **92,** 55–62.
23. Merryman, J. I., DeWille, J. W., Werkmeister, J. R., Capen, C. C., and Rosol, T. J. (1994). *Endocrinology* **134,** 2424–2430.
24. Lawrence, D. A., Pircher, R., and Julien, P. (1985). *Biochem. Biophys. Res. Commun*. **133,** 1026–1034.
25. Lyons, R. M., Keski-Oja, J., and Moses, H. L. (1988). *J. Cell Biol*. **106,** 1659–1665.
26. Lawrence, D. A., Pircher, R., Kryceve-Martinerie, C., and Julien, P. (1984). *J. Cell. Physiol*. **121,** 184–188.
27. Kaiser, S. M., Laneuville, P., Bernier, S. M., Rhim, J. S., Kremer, R., and Goltzman, D. (1992). *J. Biol. Chem*. **267,** 13,623–13,628.
28. Blomme, E. A. G., Weckmann, M. T., Capen, C. C., and Rosol, T. J. (1997). *Exp. Cell. Res*. **238,** 204–215.
29. Poumay, Y. and Pittelkow, M. R. (1995). *J. Invest. Dermatol*. **104,** 271–276.
30. Karasek, M. A. and Liu, S. C. (1975). *J. Invest. Dermatol*. **65,** 336–358.
31. Eckert, R. L., Crish, J. F., and Robinson, N. A. (1997). *Physiol. Rev*. **77,** 397–424.
32. Gibson, D. F. C., Ratnam, A. V., and Bikle, D. D. (1996). *J. Invest. Dermatol*. **106,** 154–161.
33. Pillai, S. and Bikle, D. (1991). *J. Cell. Physiol*. **146,** 94–100.
34. Kruszewski, F. H., Hennings, H., Yuspa, S. H., and Tucker, R. W. (1991). *Am. J. Physiol*. **261,** C767–C773.
35. Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K., and Yuspa, S. H. (1980). *Cell* **19,** 245–254.
36. Yuspa, S. H., Kilkenny, A. E., Steinert, P. M., and Roop, D. R. (1989). *J. Cell. Biol*. **109,** 1207–1217.
37. Millan, F. A., Denhez, F., Kondaiah, P., and Akhurst, R. J. (1991). *Development* **111,** 131–144.
38. Wilke, M. S., Hsu, B. M., Wille, J. J., Jr., Pittelkow, M. R., and Scott, R. E. (1988). *Am. J. Pathol*. **131,** 171–181.
39. Matsumoto, K., Hashimoto, K., Hashiro, M., Yoshimasa, H., and Toshikawa, K. (1991). *J. Cell. Physiol*. **145,** 95–101.
40. Herzinger, T., Wolf, D. A., Eick, D., and Kind, P. (1995). *Oncogene* **10,** 2079–2084.
41. Holick, M. F., Chen, M. L., Kong, X. F., and Sanan, D. K. (1996). *J. Invest. Dermatol*. **S1,** 1–9.
42. Stewart, A. F., Horst, R., Deftos, L. J., Cadman, E. C., Lang, R., and Broadus, A. E. (1980). *N. Engl. J. Med*. **303,** 1377–1383.
43. Burtis, W. J., Brady, T. G., Orloff, J., Ersbak, J. B., Warrel, R. P., Jr., Olson, B. R., et al. (1990). *N. Engl. J. Med*. **322,** 1106–1112.
44. Wysolmerski, J. J., Vasavada, R., Foley, J., Weir, E. C., Burtis, W. J., Kukreja, S. C., et al. (1996). *Cancer Res*. **56,** 1043–1049.

45. Munger, J. A. P., Pittelkow, M. R., Holt, J. T., and Moses, H. L. (1992). *Cell Growth Differ*. **3,** 291–298.
46. Reiss, M. and Stash, E. B. (1990). *Cancer Commun*. **2,** 363–369.
47. Henderson, J., Sebag, M., Rhim, J., Goltzman, D., and Kremer, R. (1991). *Cancer Res*. **51,** 6521–6528.
48. Boyce, S. T. and Ham, R. G. (1985). *J. Tissue Cult Methods* **9,** 83–93.
49. Rosol, T. J., Steinmeyer, C. L., McCauley, L. K., Merryman, J. I., Werkmeister, J. R., Groene, A., et al. (1993). *Vet. Immunol. Immunopathol*. **35,** 321–337.
50. Cone, J. L., Brown, D. R., and DeLarco, J. E. (1988). *Anal. Biochem*. **168,** 71–74.
51. Chomczynski, P. and Sacchi, N. (1987). *Anal. Biochem*. **162,** 156–160.
52. McCauley, L. K., Beecher, C. A., Melton, M. E., Werkmeister, J. R., Jueppner, H., Abou-Samra, A. B., et al. (1994). *Mol. Cell. Endocrinol*. **101,** 331–336.
53. Vasavada, R. C., Wysolmerski, J. J., Broadus, A. E., and Philbrick, W. M. (1993). *Mol. Endocrinol*. **7,** 273–282.
54. Mangin, M., Ikeda, K., and Broadus, A. E. (1990). *Gene* **95,** 195–202.
55. Jiang, C., Connolly, D., and Blumenberg, R. (1991). *J. Invest. Dermatol*. **97,** 969–973.