

## Parathyroid Hormone-Related Protein Production by Normal Human Keratinocytes *in Vitro*

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The role of parathyroid hormone-related protein (PTHrP) and its regulation in normal epidermal physiology is not currently known. Recent evidence suggests that PTHrP production may be related to the degree of differentiation of keratinocytes *in vitro*. This investigation characterized the production of PTHrP by normal human foreskin keratinocytes (NHFK) during both spontaneously occurring and induced differentiation *in vitro*. PTHrP production in keratinocyte serum-free conditioned medium was determined using an N-terminal radioimmunoassay for human PTHrP (1-36). Agents known to stimulate (calcium, 1,25-dihydroxyvitamin D<sub>3</sub>) or inhibit (transforming growth factor- $\beta$ ) keratinocyte differentiation were examined for their ability to alter production of PTHrP. Measurements of cell number and involucrin content of the cultures were made to confirm the effects of these agents on keratinocyte growth and differentiation. The production of PTHrP in control cultures (under low calcium conditions, 0.08 mM) was decreased and involucrin content increased ( $P < 0.01$ ) after the cells became confluent. The addition of 1 mM calcium to keratinocyte medium increased cell number and involucrin content of the cultures ( $P < 0.05$ ) but inhibited production of PTHrP ( $P < 0.01$ ). The 1,25-dihydroxyvitamin D<sub>3</sub> (10 nM) had no significant effect on cell number or PTHrP production, but increased involucrin content ( $P < 0.05$ ). Transforming growth factor- $\beta$  (5 ng/ml) decreased both cell number ( $P < 0.05$ ) and involucrin content ( $P < 0.01$ ), but significantly stimulated PTHrP production ( $P < 0.01$ ). These data indicate that the production of PTHrP by NHFK is inhibited with the onset of both spontaneously occurring and calcium-induced differentiation *in vitro*, while transforming growth factor- $\beta$  inhibited differentiation and upregulated PTHrP production in normal human keratinocytes. © 1993 Academic

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### INTRODUCTION

Parathyroid hormone-related protein (PTHrP) is a recently described hormone that can bind to parathyroid hormone (PTH) receptors and stimulate osteoclastic bone resorption and renal reabsorption of calcium [1]. PTHrP was originally isolated from tumors of patients with humoral hypercalcemia of malignancy (HHM) and is one of the primary etiologic agents in the pathogenesis of the disease [2]. Humans and animals [3-5] with HHM have increased circulating levels of PTHrP. In addition, the clinical signs of HHM have been reversed in a nude mouse model of HHM by administration of anti-PTHrP antibody [6].

PTHrP also has been identified in many normal tissues including endocrine glands, lactating mammary gland, brain, placenta, and skin [7-12]. In contrast to patients with HHM, PTHrP circulates in very low levels ( $<1$  pM) in normal adult humans and animals which suggests that PTHrP acts as a paracrine or autocrine hormone in most tissues. The role of PTHrP in most normal tissues and the regulation of its synthesis and secretion is poorly understood at present.

Normal human keratinocytes were selected to investigate the control of PTHrP production since both normal and neoplastic keratinocytes have been shown to produce PTHrP and HHM may be associated with squamous cell carcinomas [2, 11]. In addition, PTHrP production by normal, transformed, and neoplastic keratinocytes has been demonstrated *in vitro* [13-16]. In normal epithelium, PTHrP is thought to have biologic effects that are distinct from the PTH-like endocrine effects associated with HHM and may be involved in the regulation of epithelial growth and differentiation [13, 14, 17].

Recent studies of PTHrP production and secretion by normal and malignant keratinocytes have suggested a relationship between the differentiated state of the cell and the production of PTHrP. Kremer *et al.* [13] using normal human keratinocytes derived from breast reduction tissue demonstrated increased PTHrP produc-

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tion after treatment with calcium. In addition, treatment with 1,25-dihydroxyvitamin D<sub>3</sub> initially decreased and then increased PTHrP production. These data suggested that PTHrP production was enhanced with the induction of keratinocyte differentiation. In contrast, Löwik *et al.* [14] found that PTHrP production was inhibited by the addition of calcium in studies using normal neonatal or malignant keratinocytes cocultured with irradiated 3T3 fibroblasts. In addition, the production of PTHrP was observed to be greater in cells derived from less-differentiated squamous cell carcinomas than that in normal keratinocytes.

The purpose of this study was to characterize the production of PTHrP by normal human foreskin keratinocytes during spontaneous and induced differentiation *in vitro*. Calcium, 1,25-dihydroxyvitamin D<sub>3</sub>, and transforming growth factor- $\beta$  (TGF- $\beta$ ) were added to the cell culture medium in order to determine their effects on PTHrP production and keratinocyte growth and differentiation.

## MATERIALS AND METHODS

**Chemicals and supplies.** Keratinocyte serum-free medium (SFM) was purchased from Gibco BRL Life Technologies Inc. (Grand Island, NY). Human PTHrP (1-36) was purchased from Bachem California (Torrance, CA). The 1,25-dihydroxyvitamin D<sub>3</sub> was prepared as a 10 mM stock solution in absolute ethanol and was generously provided by Hoffman-LaRoche Inc. (Nutley, NJ). Calcium chloride was prepared as a 0.1 M stock in sterile water. Transforming growth factor- $\beta$  derived from human platelets was purchased from R&D Systems (Minneapolis, MN) and was prepared as a 1  $\mu$ g/ml stock solution in 4 mM HCl and 1 mg/ml bovine serum albumin (BSA). TGF- $\beta$  and 1,25-dihydroxyvitamin D<sub>3</sub> were stored protected from light at -20°C and calcium was stored at 4°C. All reagents were diluted to final concentration in cell culture medium just prior to use. Sodium [<sup>125</sup>I]iodide was purchased from NEN (DuPont, Boston, MA). All other chemicals were of reagent grade and purchased from standard suppliers.

**Keratinocyte cell culture.** Serum-free culture of normal human foreskin keratinocytes (NHFK) was conducted as previously described by Boyce and Ham [18]. Briefly, neonatal foreskin tissues were cut into small pieces and incubated for 48 h in 0.2% trypsin (type III, Sigma Chemical Co., St. Louis, MO) at 4°C. The epidermis was then removed from the underlying dermis and dispersed in MCDB 153 (Sigma) medium containing 10% fetal bovine serum (Gibco) in order to inactivate the trypsin. The cell suspension was centrifuged, and the pellet was resuspended in SFM containing low calcium (0.08 mM), placed in 75-cm<sup>2</sup> tissue culture flasks, and incubated at 37°C. The medium was replaced in stock cultures every 48 h and cells passaged at a ratio of 1:10 at 70% confluence.

**Collection of keratinocyte-conditioned medium.** All experiments (except the effect of serial passage on PTHrP production) were completed with NHFK at passages 3-5 using 12-well (3.83 cm<sup>2</sup>) tissue culture plates. Newly established cultures were incubated for 48 h at 37°C. Following this incubation, the medium was removed and the cultures were washed with calcium- and magnesium-free Dulbecco's phosphate-buffered saline (DPBS). Fresh medium containing the appropriate treatment (calcium, 1,25-dihydroxyvitamin D<sub>3</sub>, TGF- $\beta$ , or vehicle control) was added at this time. Sample collections were made over multiple time points in all experiments. Unless otherwise noted, treatments were added with each subsequent change of media at 48-h intervals and collection of keratinocyte serum-free conditioned medium was made after 24 h of incubation at the time of termination of

the cultures. Conditioned medium was stored at -20°C until assayed for PTHrP content.

**Involucrin determination.** Involucrin determinations were made using an enzyme-linked immunosorbent assay (ELISA) kit (BTI, Stoughton MA). Following the collection of conditioned medium, cells were scraped into 1 ml Hanks' balanced salt solution and frozen at -20°C. Samples were sonicated in buffer (20 mM Tris-HCl, 2 mM EDTA, pH 7.4). Involucrin content of the cell culture sonicate was measured on 1:10, 1:100, and 1:1000 dilutions of the cell sonicates. This assay was sensitive to 0.5 ng involucrin/ml of sonicate.

**Protein determination.** Protein determinations were made using a BCA kit (Pierce Biochemical, Rockford, IL) adapted for use with a microtiter plate reader (Molecular Devices, Menlo Park, CA). Bovine serum albumin standards ranged from 20 to 1200  $\mu$ g/ml.

**Cell enumeration.** Total cell counts were completed using a hemocytometer and the trypan blue dye exclusion method.

**Nonequilibrium radioimmunoassay for PTHrP.** A radioimmunoassay (RIA) was used to measure immunoreactive human N-terminal PTHrP (1-36) in NHFK culture medium as previously described by our laboratory [19]. The RIA is sensitive to 0.1 ng PTHrP/ml of conditioned medium. Briefly, standard and unknown (conditioned medium) samples were incubated with 100  $\mu$ l of polyclonal chicken anti-human PTHrP antibody (10 mg IgG/ml, diluted 1:1000 in 20 mM sodium phosphate buffer, pH 7.4) for 48 h at 4°C. Standards contained known amounts of PTHrP (1-36) ranging from 0 to 500 pg PTHrP/100  $\mu$ l sample. Following the first incubation, high-performance liquid chromatography-purified [<sup>125</sup>I]-PTHrP (1-36<sup>Tyr</sup>) (10,000 cpm/tube) [20] was added to all tubes, vortexed and incubated for an additional 48 h at 4°C. Assay controls included tubes measuring both total counts and nonspecific binding of [<sup>125</sup>I]-PTHrP (1-36<sup>Tyr</sup>). After 48 h incubation with [<sup>125</sup>I]-PTHrP (1-36<sup>Tyr</sup>), antigen-antibody complex was separated from unbound antibody by the addition of 1 ml 20 mM sodium phosphate buffer containing dextran-coated activated charcoal (8 mg/ml charcoal, 0.8 mg/ml dextran). Tubes were vortexed and centrifuged for 45 min at 3000 rpm at 4°C. The supernatant was transferred to clean tubes and counted in a gamma counter. The presence of immunoreactive PTHrP in the conditioned medium was identified by inhibition of [<sup>125</sup>I]-PTHrP (1-36<sup>Tyr</sup>) binding, calculated by log-logit transformation, and plotted against the standard curve using Sceria 1.0 (Packard Instr. Co., Downers Grove, IL).

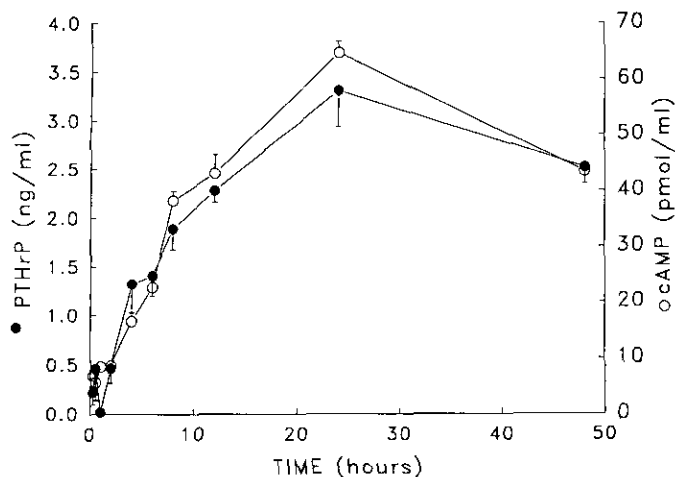
**Assay for PTHrP-associated biologic activity.** PTHrP-associated biologic activity of keratinocyte-conditioned medium was determined by measurement of adenylate cyclase stimulation in rat osteoblast-like cells (ROS 17/2.8) as previously described by our laboratory [21]. Briefly, 500  $\mu$ l of conditioned medium was added in triplicate to wells of confluent ROS 17/2.8 cells containing 1 ml Dulbecco's modified Eagle's medium/1 mM isobutylmethylxanthine (a phosphodiesterase inhibitor) and incubated for 10 min at 37°C. The reaction was stopped with the addition of 50  $\mu$ l 1.2 M trichloroacetic acid. Samples were frozen overnight at -20°C in order to extract the cAMP and centrifuged for 10 min at 1200 rpm. The supernatant was neutralized with 4 N KOH and the cAMP quantified by radioreceptor assay [22].

**Statistical analysis.** All samples were analyzed in triplicate and compared to appropriate controls by either Student's *t* test or one-way analysis of variance using the statistical program Instat (GraphPAD Software, San Diego, CA). PTHrP content of the keratinocyte-conditioned medium was expressed as nanograms per milliliter (mean  $\pm$  standard error of the mean, SE). In experiments where cell counts were made, PTHrP and involucrin were normalized to total cell number and expressed as nanograms/cell  $\times 10^{-6}$  (mean  $\pm$  SE).

## RESULTS

### *Effect of Serial Passage of Keratinocytes on PTHrP Secretion*

NHFK were cultured in triplicate 25-cm<sup>2</sup> tissue culture flasks containing 5 ml SFM. Conditioned medium



**FIG. 1.** Comparison of immunoreactive and biologically active (adenylate cyclase stimulation) PTHrP content of normal human foreskin keratinocyte-conditioned medium.

was collected and replaced with fresh medium at 48-h intervals. Cellular confluence at each interval was determined by phase-contrast microscopy and cells were passaged at 70% confluence. Growth rate of the keratinocytes and production of PTHrP were followed longitudinally over eight consecutive passages of the keratinocyte cultures.

Both the rate of keratinocyte growth and the amount of immunoreactive PTHrP detected in the conditioned medium decreased with increasing passage number. The growth rate (time to reach 70% confluence) of NHFK increased over the first several passages and then decreased markedly at passages 7 and 8. Over the first seven passages, NHFK exhibited clonal growth but by the eighth passage keratinocyte cultures were no longer growing in colonies and failed to reach confluence. Changes in PTHrP production over serial passages paralleled the changes seen in the rate of keratinocyte growth. NHFK at first passage produced PTHrP in relatively low quantities which ranged from  $289 \pm 35$  pg/ml at the beginning of culture to  $1133 \pm 296$  pg/ml just prior to passage. At passage 5, NHFK PTHrP production was maximal and ranged from  $575 \pm 94$  pg/ml at the beginning of culture to  $4131 \pm 538$  pg/ml just prior to passage. By passage 8, PTHrP was produced by NHFK in amounts which ranged from  $274 \pm 14$  pg/ml at the beginning of culture to  $546 \pm 260$  pg/ml at the termination of the cultures.

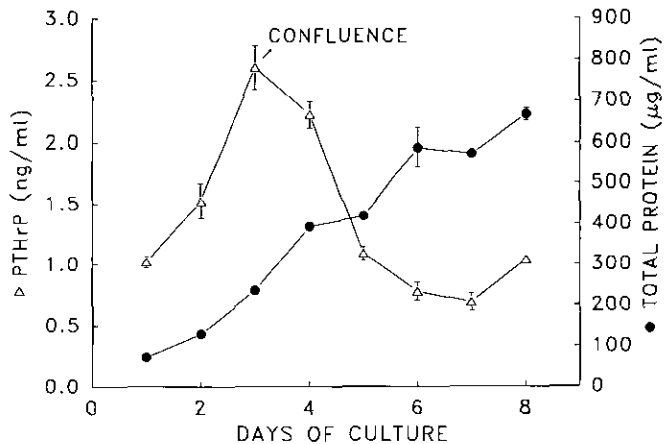
#### *Comparison of Immunoreactive and Bioactive PTHrP Accumulation in Keratinocyte Conditioned Medium*

This experiment was undertaken in order to determine the rate of accumulation and the stability of PTHrP in keratinocyte-conditioned medium between medium changes and to confirm that immunoreactive

PTHrP (quantified by RIA) represented biologically active protein (as measured by adenylate cyclase stimulation in ROS 17/2.8 cells). NHFK were established at third passage in 12-well tissue culture plates. Medium collections from triplicate wells of 90% confluent cultures were made after 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h of incubation at 37°C. Conditioned medium samples were then analyzed for immunoreactive PTHrP by radioimmunoassay and biologically active PTHrP by adenylate cyclase stimulation assay. Identical increases in the immunoreactive and bioactive PTHrP content of keratinocyte-conditioned medium were observed during the 48 h of incubation (Fig. 1). Both immunoreactive and biologically active PTHrP contents were maximal after 24 h of incubation.

#### *Effect of Cell Density on PTHrP Secretion*

The PTHrP content of NHFK-conditioned medium was examined over 8 days in cultures established at subconfluent cell density in order to determine the effect of cell density on PTHrP production. Keratinocytes at third passage were established at 20% confluency in 12-well tissue culture plates. Conditioned medium and 0.1 N NaOH cell culture lysates were collected from triplicate wells as previously described every 24 h for determination of PTHrP and total protein concentrations, respectively (Fig. 2). PTHrP content of the conditioned medium increased over the first 3 days of the experiment and was maximal at confluence (Day 3). PTHrP content was significantly decreased ( $P < 0.01$ ) on Days 5–8 compared to that on Day 3. Total protein content of the cell culture lysates increased linearly during the culture period and was significantly increased ( $P < 0.01$ ) on Days 3–8 of the study, indicating that the cells remained metabolically active.



**FIG. 2.** The effect of cell density on PTHrP production and total protein content of normal human foreskin keratinocytes (NHFK).

TABLE 1

Effects of Continuous Treatment with Calcium, 1,25-Dihydroxyvitamin D<sub>3</sub> (1,25-(OH)<sub>2</sub>D<sub>3</sub>), or TGF-β on Cell Number of Normal Human Foreskin Keratinocytes

	Control (×10 <sup>5</sup> )	Treatment (×10 <sup>5</sup> )
Calcium (1 mM)		
Day 3	4.82 ± 0.32	5.35 ± 0.30
Day 7	6.50 ± 0.50	9.20 ± 0.79*
1,25-(OH) <sub>2</sub> D <sub>3</sub> (10 nM)		
Day 3	4.30 ± 0.15	4.25 ± 0.25
Day 7	5.32 ± 0.50	3.74 ± 1.32
TGF-β (5 ng/ml)		
Day 3	7.30 ± 1.00	3.62 ± 0.66*
Day 5	10.34 ± 0.50	3.55 ± 0.15*

Note. Data are expressed as means ± SE.

\* Different from control ( $P < 0.05$ ).

#### Effects of Continuous Treatment with Calcium or 1,25-Dihydroxyvitamin D<sub>3</sub> on PTHrP Secretion and Involucrin Content of NHFK

Cultures of NHFK at fourth passage were established at 30% confluency in 12-well tissue culture plates containing 1 ml medium/well. The effects of continuous treatment with calcium (1 mM) or 1,25-dihydroxyvitamin D<sub>3</sub> (10 nM) for a 7-day experimental period on PTHrP production was evaluated. Conditioned medium samples were collected from quadruplicate wells for measurement of immunoreactive PTHrP content at Days 3 and 7. In addition, cell counts and involucrin determinations were made as measures of cell proliferation and differentiation, respectively.

At Day 7, the calcium-treated NHFK cultures contained a significantly greater number of cells than control cultures ( $P < 0.05$ ) (Table 1). PTHrP content of the conditioned medium was significantly decreased by the continuous addition of calcium to the cell cultures ( $P < 0.01$ , Fig. 3). This inhibition was seen after 3 and 7 days of treatment. Calcium treatment also resulted in a significant increase in involucrin content of the cell culture sonicates as well as visible evidence of squamous differentiation (Figs. 4, 6A, and 6B).

There were no significant effects of 1,25-dihydroxyvitamin D<sub>3</sub> on cell growth or PTHrP production after 3 or 7 days of treatment (Table 1, Fig. 3). The 1,25-dihydroxyvitamin D<sub>3</sub> treatment resulted in dense colonies of polygonal cells with decreased cytoplasmic area (Fig. 6C). Involucrin content of 1,25-dihydroxyvitamin D<sub>3</sub>-treated cultures was increased ( $P < 0.05$ ) after 7 days to a level that was 50% greater than that of control cultures (Fig. 4).

#### Effect of TGF-β on PTHrP Production by NHFK

Cultures of NHFK at third passage were established at 30% confluency in 12-well tissue culture plates. The

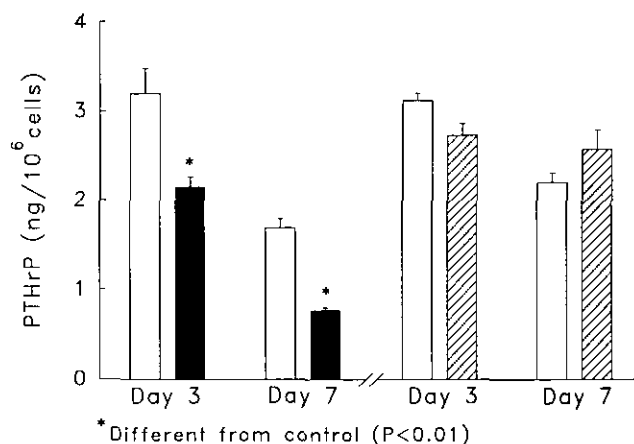


FIG. 3. The effect of calcium (1 mM, ■) or 1,25-dihydroxyvitamin D<sub>3</sub> (10 nM, ▨) on PTHrP production by normal human foreskin keratinocytes (NHFK) (□, vehicle control).

effects of continuous treatment with 5 ng/ml TGF-β on PTHrP production, and cell growth and differentiation were examined at 3 and 5 days. Conditioned media were collected from quadruplicate wells for measurement of immunoreactive PTHrP content. In addition, cell counts and involucrin determinations were made as measures of cell proliferation and differentiation, respectively.

While the addition of 5 ng/ml TGF-β significantly inhibited cell number after 3 and 5 days of treatment (Table 1), the PTHrP content of conditioned medium from TGF-β treated cultures was significantly greater ( $P < 0.01$ ) than that in control cultures during the same period (Fig. 5). Involucrin content of cell culture sonicates was significantly less than controls after 3 and 5 days of TGF-β treatment (Fig. 5). Treatment of NHFK

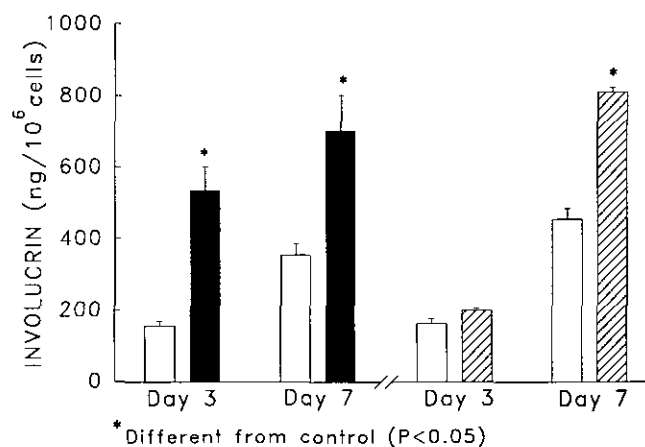
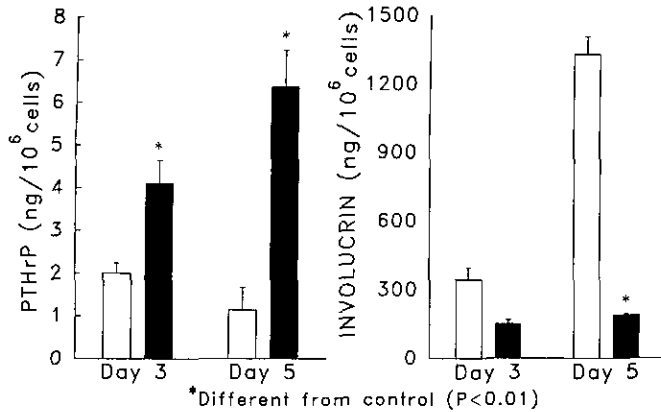


FIG. 4. The effect of calcium (1 mM, ■) or 1,25-dihydroxyvitamin D<sub>3</sub> (10 nM, ▨) on involucrin content of normal human foreskin keratinocytes (NHFK) (□, vehicle control).



**FIG. 5.** The effects of TGF- $\beta$  (5 ng/ml, ■) on PTHrP production and involucrin content in normal human foreskin keratinocytes (NHFK) (□, vehicle control).

with TGF- $\beta$  resulted in enlarged cells with granular cytoplasm (Fig. 6D).

#### DISCUSSION

The present study demonstrated that normal human foreskin keratinocytes (NHFK) produce and secrete PTHrP *in vitro* in a regulated manner. Variables normally encountered during cell culture, such as cell passage number and cellular confluence, had pronounced effects on PTHrP production by keratinocytes *in vitro*. Confluency was examined in NHFK as it related to both the production of PTHrP and the acquisition of a differentiated phenotype. PTHrP content of keratinocyte-conditioned medium increased during proliferation of the cells and decreased dramatically after confluence. Confluence of NHFK *in vitro* has been associated with terminal differentiation and simulates differentiation of skin *in vivo*. Therefore, decreased PTHrP production by NHFK after confluence may represent an important control mechanism that occurs during differentiation. Since the degree of cellular confluence affected PTHrP content of the conditioned medium, care was taken in order to avoid misinterpretation of cell density-dependent effects as direct actions of agents that may alter the growth of keratinocytes *in vitro*.

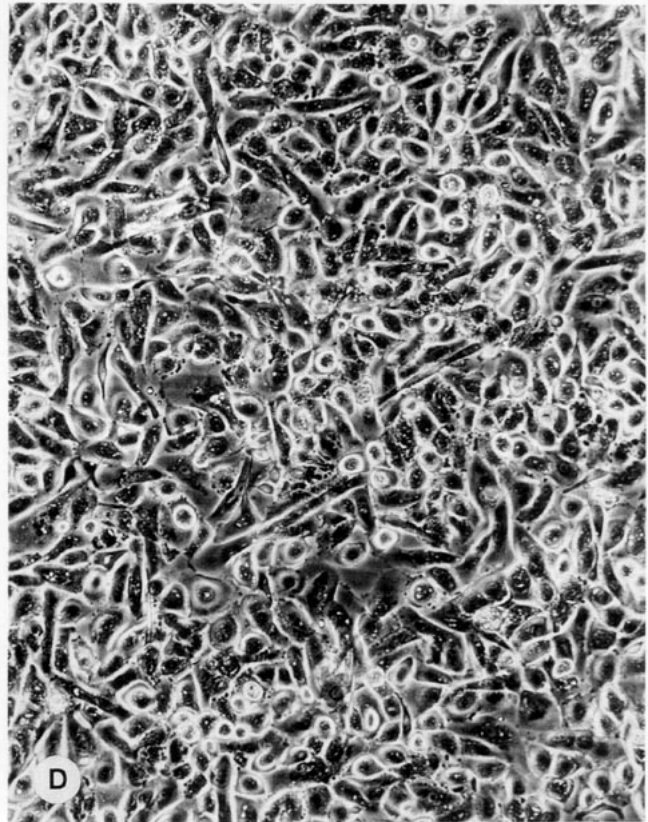
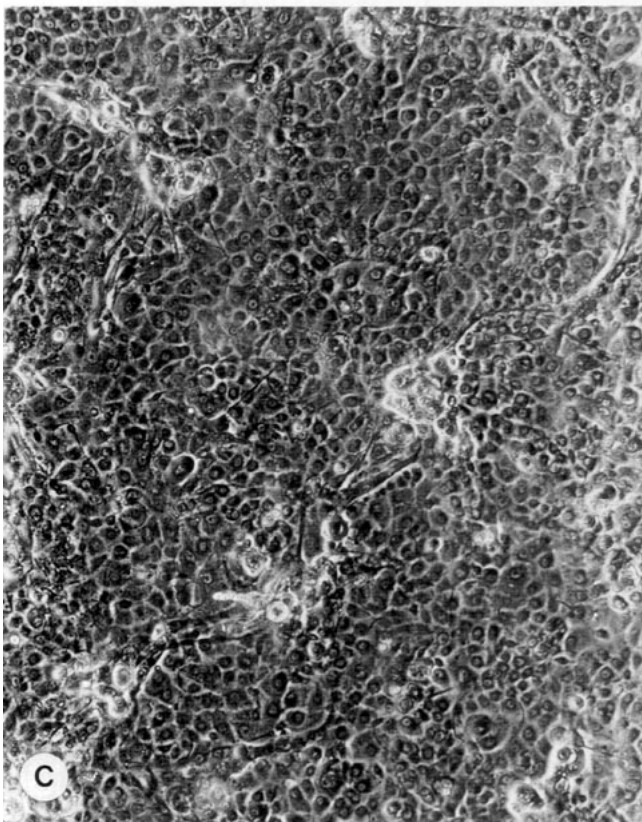
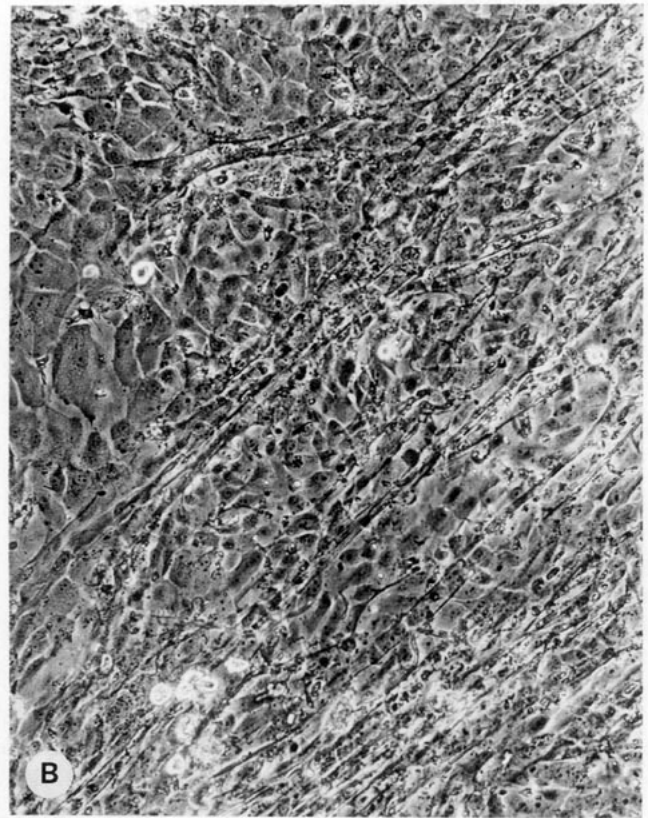
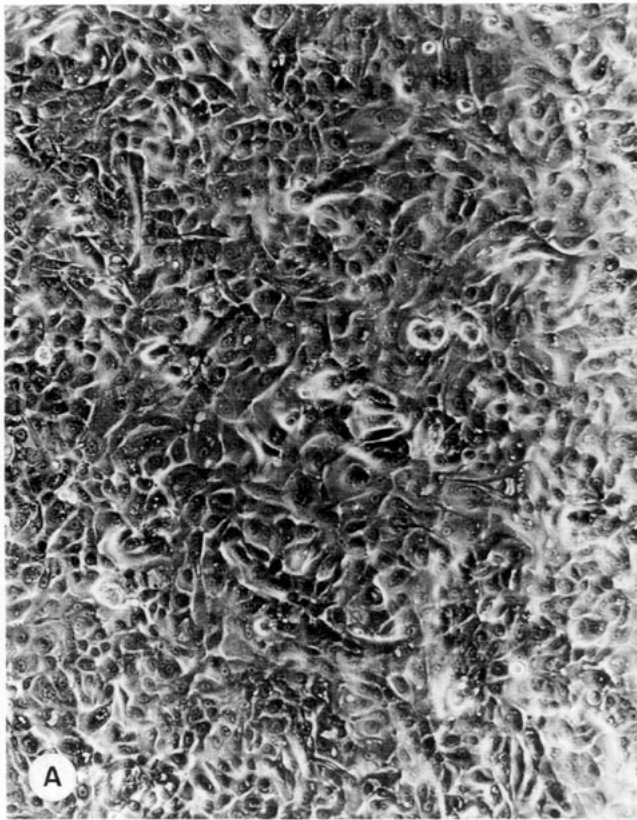
The association of increased PTHrP production by NHFK with a less differentiated keratinocyte phenotype was further corroborated by experiments which in-

involved treatment of NHFK with calcium. An increase in the extracellular calcium concentration of keratinocyte cell culture medium has been previously demonstrated to increase keratinocyte cell number and differentiation [23, 24]. Likewise, increasing the extracellular calcium concentration of NHFK medium in our investigation stimulated cell growth and differentiation, but inhibited the production of PTHrP. The effects of calcium on PTHrP production by NHFK are consistent with those of Löwik *et al.* [14] in which it was demonstrated that PTHrP production by both a squamous cell carcinoma cell line (SCC-4) and NHFK was greatest in cultures grown under low calcium (<0.1 mM) conditions. In contrast, Kremer *et al.* [13] reported that calcium stimulated PTHrP production after 8 days of treatment. This seemingly conflicting result may be explained by differences in the source of tissue used for keratinocyte cultures (adult breast reduction tissue vs neonatal foreskin tissue), culture conditions, and timing of medium collection.

The 1,25-dihydroxyvitamin D<sub>3</sub> treatment resulted in stimulation of keratinocyte differentiation only after 7 days of treatment. This observation is consistent with the findings of Pillai and Bikle [25] who found that short-term exposure of normal keratinocytes to 1,25-dihydroxyvitamin D<sub>3</sub> failed to stimulate intracellular calcium and cornified envelope formation (markers of keratinocyte differentiation). They also reported that increasing the extracellular calcium concentration of the cell culture media resulted in immediate increases in intracellular calcium and cornified envelope formation. The authors suggested that the effects of calcium and 1,25-dihydroxyvitamin D<sub>3</sub> on keratinocyte differentiation were mediated by their ability to increase intracellular calcium. In our study 1,25-dihydroxyvitamin D<sub>3</sub> had no effect on PTHrP production by keratinocytes. Differences in the mechanism of calcium- and 1,25-dihydroxyvitamin D<sub>3</sub>-induced differentiation may account for this apparent difference. In contrast, Kremer *et al.* [13] found that 1,25-dihydroxyvitamin D<sub>3</sub> treatment resulted in early (48 h) inhibition of PTHrP production which was followed by stimulation of PTHrP production after several days of treatment.

TGF- $\beta$  has been shown to inhibit the growth and differentiation of human keratinocytes *in vitro* under low calcium conditions [26]. This observation was confirmed in our experiments and extended to demonstrate

**FIG. 6.** Phase-contrast photomicrographs of NHFK (80 $\times$  magnification) illustrating the morphologic changes associated with the following treatments: (A) Control cultures of normal human foreskin keratinocytes exhibited a predominantly polygonal morphology characterized by solid sheets or colonies of epithelial cells. (B) Treatment with calcium (1 mM) resulted in squamous differentiation of NHFK as evidenced by stratification of elongated cells and the formation of large squamous cells. (C) Treatment with 1,25-dihydroxyvitamin D<sub>3</sub> (10 nM) resulted in confluent monolayers of NHFK consisting of tightly packed polygonal cells with decreased cytoplasmic area and a high nuclear to cytoplasmic ratio. (D) Treatment with TGF- $\beta$  (5 ng/ml) resulted in large elongated, polygonal, or round cells that were randomly arranged and exhibited cytoplasmic granularity and vacuolation.





that TGF- $\beta$  also resulted in marked stimulation of PTHrP production. However, this may be due to a direct effect of TGF- $\beta$  on increasing the expression of PTHrP mRNA and not related to the effects of TGF- $\beta$  on NHFK proliferation and differentiation [27, 28]. Stimulation of PTHrP production by TGF- $\beta$  *in vitro* has also been reported in a canine oral squamous cell carcinoma cell line and human uterine cells [16, 27]. TGF- $\beta$  is often coproduced with PTHrP by neoplasms associated with humoral hypercalcemia of malignancy [29] and may act by stimulating increased production of PTHrP by such tumors. The interaction of PTHrP and TGF- $\beta$  may be important in the physiology of normal skin [26] and in the pathogenesis of humoral hypercalcemia of malignancy [2].

Fibroblast contamination was not observed in the NHFK cultures of this investigation and the likelihood of such contamination was minimized by differential trypsinization and the use of serum-free selective medium. Hoekman *et al.* [15] found that NHFK and certain squamous cell carcinomas grown in serum-containing medium increased their production of PTHrP in response to the addition of fibroblasts to the cultures.

In summary, these results indicate that PTHrP production and secretion by normal human keratinocytes during naturally occurring and induced differentiation *in vitro* is associated with a less differentiated phenotype. PTHrP appears to act as a paracrine factor during normal growth and differentiation of the epidermis. In contrast, tumors derived from keratinocytes have abnormal regulation of PTHrP synthesis and secretion which results in the clinical syndrome of humoral hypercalcemia of malignancy.

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