Overexpression of parathyroid hormone-related protein in the skin of transgenic mice interferes with hair follicle development

(epithelial–mesenchymal interactions/organogenesis/dorsal–ventral patterning/humoral hypercalcemia of malignancy)

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ABSTRACT Parathyroid hormone-related peptide (PTHrP) was initially discovered as the cause of the syndrome of humoral hypercalcemia of malignancy. Subsequently, the PTHrP gene has been shown to be expressed in a wide variety of normal tissues, including skin. Because the biological function of PTHrP in skin remains unknown, we used the human keratin 14 promoter to target overexpression of PTHrP to the skin of transgenic mice. We achieved a 10-fold level of overexpression in skin, and human keratin 14 promoter–PTHrP transgenic mice displayed a disturbance in normal hair follicle development. These mice either failed to initiate follicle development or showed a delay in the initiation of follicles. These findings suggest that PTHrP normally plays a role in the early stages of hair follicle development and support previous speculation that the peptide may function in regulating cellular differentiation.

Parathyroid hormone (PTH)-related peptide (PTHrP) was initially discovered by its production from tumors associated with the clinical syndrome of humoral hypercalcemia of malignancy (1). Subsequently, PTHrP and its mRNA have been detected in a wide array of normal fetal and adult tissues, leading to speculation that it acts as a paracrine or autocrine factor involved in the regulation of growth and/or differentiation in these sites (2–4). Human keratinocytes in primary culture were the first normal cells shown to secrete PTHrP (5). Immunohistochemical studies have localized PTHrP from the basal layer through the granulosal layer of the epidermis, in skin appendages, and in fetal rat skin as early as 14 days of gestation (4, 6). PTHrP gene expression is regulated in cultured keratinocytes by serum, by 1,25-dihydroxyvitamin D, and by an as-yet-unidentified dermal fibroblast-derived factor (7, 8). The classical PTH/PTHrP receptor is present on dermal fibroblasts (9), but there is also evidence for specific PTHrP receptors on keratinocytes (10). Thus, PTHrP is produced in skin; its production is regulated, and its effects in skin may be mediated by autocrine or paracrine pathways, or both.

Despite these observations, very little has been learned of the actual function of PTHrP in skin. Data suggest that PTHrP is involved in the regulation of keratinocyte proliferation, but different studies have reported conflicting results (11, 12). There is also evidence that PTHrP stimulates adenylate cyclase activity and fibronectin production in cultured dermal fibroblasts (13, 14). Recently, the human keratin 14 promoter (K14) has been used to study the role of several peptides in skin in transgenic mice (15–17). Because the pattern of transgene expression directed by this promoter overlaps with the natural distribution of PTHrP in skin, we used this strategy to overexpress PTHrP in the hope of better

defining its physiologic role in skin. In this report, we show that overexpression of PTHrP in skin profoundly affects the initiation of hair follicle development.

MATERIALS AND METHODS

Generation and Identification of Transgenic Mice. The K14–PTHrP transgene was constructed by using a K14–human growth hormone cassette as described (15). Into this vector we introduced a 568-bp *EcoRI*–*Sty* I cDNA fragment encoding the human PTHrP-(1–141) isoform. We included all pre-pro sequences but extended only 20 bp into the 3' untranslated region, truncating the A+U-rich mRNA instability sequences (18) (see Fig. 1A). Transgenic mice were identified by Southern analysis of genomic DNA prepared from tails, using either the 568-bp human PTHrP cDNA or a 153-bp *Bgl* II–*Pvu* II genomic fragment from exon 5 of the human growth hormone gene as probes.

Analysis of Transgene Expression. Total cellular RNA was prepared from full-thickness skin and assayed by RNase protection assay as described (19). The cRNA probes protected the following sequences: (i) a 349-bp band corresponding to an *Avr* II–*Pvu* II genomic fragment of mouse PTHrP gene, (ii) a 283-bp band corresponding to a *Sau*3a–*Pvu* II cDNA fragment (cDNA gift of L. Defize, Utrecht, The Netherlands) of murine PTH/PTHrP receptor gene, (iii) a 220-bp band corresponding to a *Sau*3a–*Sau*3a cDNA fragment of mouse cyclophilin gene (cDNA courtesy of J. Sutcliffe, Scripps Clinic, La Jolla, CA), (iv) a 400-bp band corresponding to a *Pst* I–*Pst* I cDNA fragment of mouse K14 gene (cDNA provided by J. Compton, National Institutes of Health, Bethesda, MD), and (v) a 307-bp band corresponding to a *Pvu* II–*Sac* I cDNA fragment of human PTHrP gene. Acid-urea extracts were prepared as described (1), and immunoreactive PTHrP was measured by immunoradiometric assay (20). Total protein concentrations were determined by the Bradford method (ref. 21; Bio-Rad).

RESULTS

PTHrP Is Overexpressed in the Skin of Transgenic Mice. We used 2 kb of human K14 to drive expression of a cDNA encoding the human 141-amino acid isoform of PTHrP (Fig. 1A) (15–17). Eleven founder mice were generated, three of whom were runted and died within 1 week of life. Of the remaining eight mice, two mosaic founders gave rise to independent true-breeding lines with identical phenotypes (lines 6 and 7).

We used RNase protection analysis of total RNA prepared from full-thickness skin to assay transgene expression. Fig. 1B shows that transgene expression was of comparable magnitude in the two individual lines, was specific to transgenic animals, and was easily distinguishable from native

FIG. 1. Characterization of transgene expression in K14-PTHrP transgenic mice. (A) Diagrammatic representation of K14-PTHrP transgene. hPTHrP, human PTHrP; hGH, human growth hormone. (B) RNase protection assay of 30 μ g of total RNA from full-thickness skin of 5-week-old transgenic (T) and normal littermate (N) mice from lines 6 and 7. The upper band represents native murine PTHrP (mPTHrP) mRNA, while the lower band represents human PTHrP (hPTHrP) mRNA (the transgene). Numbers beneath lanes represent PTHrP content in acid-urea extracts of whole-thickness skin from these same animals. prot., Protein. (C and D) PTHrP immunohistochemistry of back skin from transgenic (C) or normal littermate (D) mice. Tissue was fixed in 4% (wt/vol) paraformaldehyde, paraffin-embedded, and sectioned. The primary antiserum was affinity-purified anti-PTHrP-(37-74) diluted at 1:25; sections were developed with avidin-biotin horseradish peroxidase. ($\times 200$.)

murine PTHrP expression. There was always slightly less endogenous PTHrP mRNA expression in transgenic animals as compared with controls. Because endogenous K14 mRNA levels were not similarly reduced in transgenic skin (see Fig. 4B), PTHrP may exert some sort of feedback regulation on its own mRNA levels. As expected from previous reports (15-17), transgene expression was tissue-specific, being absent in liver and lung tissue (data not shown).

To quantitate the degree of overexpression, we measured immunoassayable PTHrP in acid-urea extracts of skin (1, 20). When corrected for total protein content, there was an approximate 10-fold increase in PTHrP content in transgenic skin (see Fig. 1B). Despite this order-of-magnitude overexpression, transgenic mice were not hypercalcemic, and we detected no PTHrP in their circulation.

Immunohistochemistry (Fig. 1C and D) of skin taken from transgenic animals demonstrated the expected localization of

FIG. 2. Adult K14-PTHrP transgenic mice. The mouse at left is an albino female from line 7; the mouse at right is an agouti female from line 6. The middle mouse is a normal female control.

PTHrP in basal keratinocytes and outer-root sheath cells of hair follicles (16, 17).

K14-PTHrP Mice Display Hair Loss. At birth, transgenic mice were indistinguishable from their littermates, but by 6 days of life they could be detected by a lack of hair eruption on their ventral surfaces. In agouti transgenic mice, this distinction could be made by 4-5 days after birth, for their ventral surface became deeply pigmented and remained so throughout life. Fig. 2 shows that adult transgenic mice were characterized by a sharply demarcated region of ventral hairlessness, extending the length of the animal and to the midaxillary lines bilaterally. There was an interesting sexual dimorphism in the pattern of hair growth; males were less severely affected and showed some ventral hair growth in two paramedian strips (not shown).

In the dorsal coat, there was a delay in the eruption of hair in transgenic mice compared with their littermates. The dorsal coat in adult transgenic mice continued to be thinner overall and was deficient in "overhairs," those long hairs seen to protrude beyond the general coat in normal mice. Analysis of hair cut or plucked from the dorsum of transgenic animals revealed that all four hair subtypes (22) were present, but all hairs were shorter and thinner than those taken from normal littermates. In addition, the relative frequency of the awl and auchene subtypes was reduced. The loss of these longer, thicker, and more heavily pigmented hair shafts most likely was responsible for the apparent lack of overhairs in the coats of transgenic mice and the lighter, more yellow coat color in agouti transgenic mice.

Histopathology. The most striking histopathological changes were found in the ventral skin of transgenic mice, where hair follicles were almost completely absent (Fig. 3A and B). The ventral epidermis in transgenic animals was thickened, and there was mild hyperkeratosis as compared with littermates. The dermis was expanded and very cellular, containing a heterogeneous collection of fibroblasts, capillaries, and scattered histiocytes but containing no follicles. Despite these changes, the overall thickness of the skin was reduced, as very little fat was present in the subcutis.

The histologic findings in the dorsal skin of transgenic mice were more subtle. The transition from hairless to hairy regions was abrupt, proceeding from an absence of all follicles, through a zone of stunted, partially developed

FIG. 3. Histological sections of normal (A, D, and F) and transgenic (B, C, E, and G) mouse skin. Tissue was fixed in 10% (vol/vol) buffered formalin or 4% paraformaldehyde, paraffin-embedded, sectioned, and stained with hematoxylin/eosin. (A and B) Sections of ventral skin from 5-day-old transgenic (B) and normal littermate (A) mice ($\times 90$.) (C) Transition zone between hairless and hair-bearing regions on ventral-lateral surface of a 3-week-old transgenic female ($\times 40$.) (D and E) Sections of dorsal skin from 5-day-old transgenic (E) and normal littermate (D) mice ($\times 75$.) (F and G) Sections of tail skin from 6-week-old transgenic (G) and normal littermate (F) mice ($\times 90$).

follicles, to normal-appearing follicles in the dorsal skin (Fig. 3C). In transgenic mice, a somewhat lower density of hair follicles occurred dorsally, with a selective decrease in the larger and more deeply penetrating follicles normally seen in the subcutis of control animals (Fig. 3D and E). The interfollicular epidermis appeared normal in dorsal skin samples from transgenic animals.

In agouti transgenic mice, a collection of both melanophages and dendritic melanocytes appeared just beneath the epidermis, accounting for the hyperpigmentation seen grossly. Dendritic melanocytes could occasionally be seen clustered around partially developed hair follicles. In contrast, neither melanocytes nor melanophages were detected beneath the epidermis in the dorsal skin of these mice, suggesting that the abnormal location of the melanocytes in the ventral skin may have been from an arrest in their migration, secondary to the failure of hair follicle development.

There were also histopathological changes in ear and tail skin. The epidermis from transgenic ear was thickened symmetrically and was mildly hyperkeratotic. Tail skin revealed an effacement of the alternating ridge-like epidermal pattern normally associated with the hair follicle ostia (Fig. 3F and G). There was a corresponding loss of the normal regions of parakeratosis associated with the ostia. No histological changes occurred in tongue, esophagus, or fore-stomach, which are known to express the K14 gene and have been affected by other transgenes driven by this promoter (15–17).

Regional Expression of Transgene. Given the striking regional phenotypic changes seen in K14-PTHrP transgenic mice, we asked whether there was differential expression of

native PTHrP, the PTH/PTHrP receptor, K14, or the transgene mRNAs in a dorsal/ventral pattern. Fig. 4A shows no

FIG. 4. (A) Ventral vs. dorsal expression of murine PTHrP (mPTHrP), murine PTH/PTHrP (mPTH/PTHrP), and murine cyclophilin mRNAs. RNase protection assay was done by using 50 μ g of total cellular RNA prepared from skin harvested from the ventral (V) or dorsal (D) surfaces of 6-week-old transgenic mice (Tg) and controls (NI). Autoradiographs were analyzed for density, and bands of interest were normalized to the cyclophilin signal (included as a loading control) with MCID software (Imaging Research, St. Catharines, Canada). This technique showed no apparent increase in ventral vs. dorsal expression of endogenous PTHrP or the PTH/PTHrP receptor in transgenic and normal mice. (B) Ventral (V) vs. dorsal (D) expression of human PTHrP (hPTHrP), murine K14 (mK14), and murine cyclophilin mRNAs. RNase protection assay was done as above. Densitometric analysis revealed arbitrary units of 8.4 and 5.4 (36% change) for the mK14 band in ventral vs. dorsal samples in transgenic animals. Analysis of ventral and dorsal hPTHrP bands gave units of 4.6 and 3.4 (26% change), respectively. In contrast, in control mice scanning of ventral vs. dorsal mK14 bands gave arbitrary units of 10.0 and 10.2 (2% change).

consistent differences in the expression of native PTHrP or the PTH/PTHrP receptor in total RNA prepared from dorsal vs. ventral skin of transgenic or control animals. Nor was there any consistent dorsal/ventral difference in the expression of native murine K14 mRNA in control animals (Fig. 4*B*). In contrast, in transgenic mice there was a consistent, modest increase in levels of both the native murine K14 and transgene mRNAs in RNA from ventral skin, as compared with RNA from dorsal skin (Fig. 4*B*). These results suggested that the increase in transgene expression seen in the ventral skin of K14-PTHrP mice was secondary to an increase in endogenous K14 expression caused by the more severe pathological changes seen in the ventral skin (15, 17). These modest changes in transgene expression were not reflected at a protein level, as PTHrP immunoreactivities in acid-urea extracts of skin from ventral and dorsal skin were similar.

PTHrP Overexpression Interferes with Follicle Initiation. To determine whether the changes in hair follicles in transgenic mice reflected a developmental or a destructive process, we examined fetal and neonatal skin from transgenic mice and their littermates. Developing follicles were easily appreciated in sections from the ventral skin of normal newborn mice but were totally absent from similar sections from transgenic mice (compare Fig. 5 *A* with *B*). In the dorsal skin of newborns, hair follicle development appeared retarded in transgenic mice compared with controls (Fig. 5 *C* and *D*). This result appeared to result from a delay in follicle initiation, as dorsal skin from day-18 embryos revealed developing follicles in normal mice with an absence of follicles in transgenic animals (data not shown), reminiscent of the picture seen in newborn ventral skin.

DISCUSSION

Hair follicle development is recognized as a paradigm of the exchange of information between the epithelium and the mesenchyme that is the fundamental basis of organogenesis (23). The first visible sign of follicle development is the aggregation of specialized mesenchymal cells at nonrandom intervals beneath the basal lamina of the fetal skin (24). This event is followed by an aggregation of epidermal cells above

the basal lamina, and these then grow into the dermis as a column of cells partially encapsulating the mesenchymal cells and inducing them to form the nascent dermal papilla (23). The papilla cells, in turn, appear to participate in the differentiation of epidermal cells to form matrix cells and eventually inner-root sheath cells and the hair shaft itself (23, 24). Although epidermal cells could contribute to induction of the mesenchymal aggregates and dermal papilla, it is clear from transplantation studies that the papilla, once formed, is sufficient to guide follicle development (25). Recently, several studies have begun to address the molecular nature of the relevant epithelial-mesenchymal interactions, and follicle development is clearly associated with a complex series of changes in membrane-associated proteins, extracellular matrix, soluble growth factors, and growth factor receptors in both cell populations (23, 24, 26).

The most striking finding in K14-PTHrP transgenic mice was the complete lack of ventral hair follicles, which apparently resulted from a primary failure of follicle induction. Therefore, overexpression of PTHrP in the basal keratinocytes of these mice would appear to interfere with the early epithelial-mesenchymal interactions that underlie the initiation of follicle development, suggesting that PTHrP normally plays a role in this process. Several observations support this interpretation. (i) Immunohistochemical studies have documented PTHrP in developing skin appendages (4), and *in situ* hybridization data have suggested the PTHrP gene is preferentially expressed in epidermal cells of developing hair pegs (27). Furthermore, PTH/PTHrP receptors have been localized to the dermal sheath and papilla of developing rodent hair follicles by *in situ* hybridization histochemistry (28). (ii) It is of interest that male transgenic mice had a less severe phenotype than females, suggesting that androgens might modulate the transgene effects. Androgens are known to exert their effects on follicle growth through their actions on dermal papilla cells (29); presumably, any modulating effects in the K14-PTHrP transgenic mice might also be mediated by these cells. (iii) Our results are similar to those recently described for transgenic mice overexpressing keratinocyte growth factor under the direction of K14 (17). Although

FIG. 5. Histological sections from ventral (*A* and *B*) or dorsal (*C* and *D*) surface of newborn transgenic mice (*A* and *C*) or normal littermates (*B* and *D*). Tissue was prepared as in Fig. 3. The arrowheads highlight developing follicles; the arrow in *D* points to a growing hair shaft. (*A*–*D* $\times 150$.)

these mice had additional epidermal changes, they also failed to initiate hair follicle growth. Keratinocyte growth factor is a member of the basic fibroblast growth factor family and is a natural product of dermal fibroblasts that specifically interacts with a splice product of the fibroblastic growth factor 2 receptor located on keratinocytes (30, 31). Given the similarity of the phenotype in these mice to those of K14-PTHrP transgenic mice, it is attractive to speculate that PTHrP and keratinocyte growth factor represent a pair of soluble factors exchanged between epidermis and mesenchyme early in the induction of hair follicle development. In this regard, it is of interest that dermal fibroblasts can modulate PTHrP secretion via a soluble factor(s) (8) and that PTHrP can stimulate fibronectin secretion and proliferation in primary cultures of neonatal dermal fibroblasts (14).

A significant aspect of the K14-PTHrP phenotype is its regionality. This aspect of the phenotype in the K14-PTHrP mice cannot be explained by dorsal/ventral differences in expression of the PTH/PTHrP receptor, and the modest increase in ventral expression of the transgene appeared secondary (i.e., it was associated with a similar increase in endogenous K14 gene expression). We think that the dorsal/ventral difference seen most likely results from regional differences in the nature of epithelial-mesenchymal interactions. A clear dorsal/ventral difference exists in the timing of hair follicle development and the resulting distribution and density of follicles in normal mice (see Fig. 3 A and D). Gradients of soluble growth factors are important in determining body patterns during development (32), and several factors thought to participate in hair follicle development, such as BMP4 and fibroblast growth factor, have been shown to operate in dorsal/ventral patterning in *Xenopus* (33). In addition, there are now several hair follicle-associated genes, including *Hoxc-8* and the *agouti* gene, that have been shown to be differentially regulated in a dorsal/ventral fashion (34–36). Therefore, we suggest that the excess PTHrP produced by the transgene interacts in a microenvironment already less conducive to hair follicle formation in the ventral skin.

PTHrP was originally discovered through its "endocrine" effects in individuals suffering from the syndrome of humoral hypercalcemia of malignancy. This paraneoplastic syndrome is probably the only circumstance in which the peptide is present in the general circulation in sufficient quantities to exert systemic effects. In fact, it is remarkable that even a 10-fold increase in the PTHrP content of the skin of these transgenic mice did not produce hypercalcemia. Because we could measure no PTHrP in their circulations, efficient mechanisms for the local destruction or sequestration of PTHrP in skin must exist.

The discovery of the widespread expression of the PTHrP gene in fetal tissues has led to speculation that the peptide may play a role in regulating cell proliferation and/or differentiation during development. The results of this organ-specific overexpression of PTHrP in transgenic mice supports such a role in hair follicle development. In addition, the recent report that PTHrP may contribute to mesoderm induction in early mouse development (37) and the preliminary report that disruption of the PTHrP gene through homologous recombination severely affects the program of differentiation in endochondral bone formation (38) suggest that PTHrP may play a more general role in development. This peptide will probably be added to the growing repertoire of soluble factors that regulate organogenesis by their paracrine interactions.

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