Parathyroid hormone-related protein induces spontaneous osteoclast formation via a paracrine cascade

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Experiments *in vivo* have established that tooth eruption fails in the absence of parathyroid hormone (PTH)-related protein (PTHrP) action in the microenvironment of the tooth because of the failure of osteoclastic bone resorption on the coronal tooth surface to form an eruption pathway. To elucidate the effects of PTHrP on osteoclast regulation in this environment, we established primary cultures of epithelial stellate reticulum cells and mesenchymal dental follicle (DF) cells surrounding the teeth. When cocultured, these cells are fully capable of supporting the formation of functional osteoclasts in the absence of added splenic osteoclast precursors, osteoblasts, or vitamin D/PTH/PTHrP. Neutralizing the effects of PTHrP resulted in a decrease in the number of osteoclasts formed, suggesting that stellate reticulum-derived PTHrP drives osteoclast formation. DF cells were found to express functional PTH/PTHrP type I receptors, and conditioned media collected from PTHrP-treated DF cells were able to induce bone resorption in the fetal-rat long-bone assay. PTHrP treatment also induced an increase in osteoclast differentiation factor expression and a concomitant decrease in osteoclastogenesis inhibitory factor expression in DF cells. The addition of osteoclastogenesis inhibitory factor resulted in a decrease in the number of osteoclasts formed in the cocultures, suggesting that osteoclast formation is mediated by osteoclast differentiation factor. Thus, PTHrP seems to regulate osteoclast formation via mediation of the DF, in a manner analogous to the osteoblast-mediated process in the peripheral skeleton. The primary coculture system of dental crypt cells also offers a system for the study of osteoclast formation and regulation.

Parathyroid hormone (PTH)-related protein (PTHrP) was discovered initially as the tumor product that is responsible for the hypercalcemia that complicates certain types of cancer. It is now known that the PTH and PTHrP genes are two members of a small gene family. One result of this common heritage is a short stretch of highly homologous sequence at the N terminus of each peptide, and another is that both products seem to be served by a single G protein-coupled receptor referred to as the type I PTH/PTHrP receptor. PTHrP is typically expressed in surface epithelia, whereas the receptor is diffusely distributed in the adjacent mesenchyme. Thus, PTHrP seems to act in a paracrine fashion (1).

Overexpression and null strategies in mice have provided convincing evidence that PTHrP is a developmental regulatory molecule. For example, overexpression of PTHrP in keratinocytes (2), mammary epithelial cells (3), and chondrocytes (4) of transgenic mice results in a developmental phenotype in each case, whereas knockout of the gene generates a chondrodystrophy that is lethal at birth (5). Our laboratory has recently rescued the PTHrP-null mouse via a genetic strategy involving crossbreeding of the PTHrP-null allele and a transgene that targets PTHrP expression to chondrocytes. The resultant rescued PTHrP-knockout mice survive to about 6 months of age but exhibit a panoply of developmental abnormalities that together constitute a generalized ectodermal dysplasia. These dysplastic features include multiple abnormalities in skin, a complete absence of mammary epithelial development, and a failure of tooth eruption (6).

Although the development of dentition in the rescued PTHrP-knockout mouse seems to be normal, early evidence of impaction can be found on histologic analysis of the first mandibular molar shortly before birth (embryonic day 18.5). By the time the first molar is scheduled to erupt (postnatal day 16), the tooth is fully encased within dense bone, in a manner similar to that seen in osteopetrotic mice (6). Unlike the situation observed in osteopetrotic mice, however, osteoclasts in the peripheral skeleton of the PTHrP knockout mouse are appropriate in number and in function, such that the defect cannot be intrinsic to osteoclasts but must be specific to the microenvironment of the tooth. Osteoclasts are recruited within the dental crypt from the rich vascular layer adjacent to the dental follicle (DF) that separates the epithelial components of the tooth (the enamel epithelium and the stellate reticulum or SR) from the surrounding alveolar bone. Thus, the crypt osteoclasts represent a separate population that is derived independently of osteoclasts elsewhere in the mandibular bone.

In the peripheral skeleton, the rates of bone formation by osteoblasts and resorption by osteoclasts are tightly coupled to maintain homeostasis of bone mass (7). In contrast, in the tooth microenvironment, some 60% of the alveolar bone surface overlying the crown is covered by osteoclasts in the virtual absence of osteoblasts at the time of initiation of eruption, allowing formation of an eruption pathway (8). After formation of the eruption pathway, osteoblastic bone formation at the base of the tooth propels it into the oral cavity (9). Thus, the process of tooth eruption represents a dramatic and unique example of completely uncoupled bone formation and resorption.

An understanding of the mechanisms governing the development of these crypt osteoclasts would not only help clarify the regulation of tooth eruption but might also be relevant to our understanding of osteoclast biology. Because osteoclasts do not seem to bear a functional PTH/PTHrP type I receptor (10), the stimulation of osteoclasts by PTH and PTHrP requires an intermediary cell that does express this receptor. In the peripheral skeleton, this role is taken by the osteoblast, but these cells are largely absent on the coronal aspect of the erupting tooth (8). Herein, we describe a series of experiments that begin to dissect

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Abbreviations: PTH, parathyroid hormone; PTHrP, PTH-related protein; DF, dental follicle; SR, stellate reticulum; IRMA, immunoradiometric assay; ODF, osteoclast differentiation factor; OCIF, osteoclastogenesis inhibitory factor; TRAcP, tartrate-resistant acid phosphatase.

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functionally the cell types and mechanisms through which PTHrP regulates the process of tooth eruption.

Materials and Methods

The culture of cells derived from rescued knockout mice, although potentially advantageous, was deemed impractical because of the difficulty of harvesting cells from the small and impacted teeth and the limitation in the number of rescued mice available. Cultures of dental tissues (SR and DF) from 8-day-old rat pups were established by using protocols that have been described in detail (11, 12). Approximately 1×10^6 epithelial cells and a similar number of DF cells can be obtained from five mandibles (the first and second molars are removed on both sides). Epithelial cells were identified by positive staining with antibodies against cytokeratins K14 (courtesy of D. Roop, Baylor College of Medicine, Houston; ref. 2) and K8/13 (Sigma; ref. 11), and DF cells were identified by staining with fibronectin (Sigma), vimentin (Roche Molecular Biochemicals), and collagen I (Sigma; ref. 12).

RNase protection assays were performed with rat PTHrP and type I PTH/PTHrP receptor probes as described (13), with cyclophilin as a loading control.

PTHrP was assayed with either a one-site RIA for PTHrP (1–34) (Peninsula Laboratories) or a two-site immunoradiometric assay (IRMA; Diagnostic Systems Laboratory) for PTHrP (1–74). cAMP was measured by RIA (Biomedical Technologies, Stoughton, MA), and the rat osteosarcoma bioassay for PTH and PTHrP was performed according to published protocols (14).

The fetal-rat long-bone assay has been described elsewhere (15). In brief, ^{45}Ca was injected into pregnant rats at day 17 after conception, and radii and ulnae from the fetuses were dissected at day 18 and allowed to equilibrate for 24 h before adding the sample to be assayed. We treated DF cells with 10 nM PTHrP (1–40) or vehicle for 24 h, washed the cells three times with 1× PBS, and then incubated them in serum-free MEM with Earle's salts supplemented with 0.1% BSA for 8 h. Conditioned media were then collected and added to the ^{45}Ca-labeled radii and ulnae for 72 h, at which time the isotope released into the medium was quantified by scintillation counting.

Tartrate-resistant acid phosphatase (TRAcP) staining (16), $\alpha_V\beta_3$ integrin staining (courtesy of J. Gailit, State University of New York, Stony Brook, NY; ref. 17), c-*src* staining (Santa Cruz Biotechnology; refs. 18 and 19), and pit formation on dentine slices (courtesy of T. Suda, Showa University, Tokyo; ref 20) were performed by using previously described protocols.

The rabbit anti-human PTHrP (1–34) antibody was obtained from Yanaihara Institute (Sizuoka, Japan), and nonimmune rabbit serum was used as a negative control (Sigma). Both were added daily to culture media at a dilution of 1:100. Osteoclastogenesis inhibitory factor (OCIF) was obtained from Alexis Corporation (San Diego) and added daily to primary cultures at a concentration of 100 nM. Mouse IgG (Bachem) was used as a control.

Northern blot analyses were performed as described (21). The osteocalcin probe was kindly provided by G. Karsenty (University of Texas, Houston; ref. 22). We were able to detect osteocalcin in 0.1 μg of total RNA from rat osteosarcoma cells treated with 10 nM vitamin 1,25 $(OH)_2$ D. The osteoclast differentiation factor (ODF; also called RANK ligand and TRANCE) and OCIF (also called osteoprotegerin) probes were provided courtesy of T. Suda and Snow Brand Milk Products (Tochigi, Japan; ref. 23). Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control (Ambion).

Results

PTHrP Is Expressed by SR Cells, and PTH/PTHrP Receptor Is Expressed by DF Cells. *In situ* hybridization of intact mouse mandible had shown that the SR and outer enamel epithelium express abundant amounts of PTHrP mRNA just before tooth eruption. The type I PTH/PTHrP receptor was primarily found on the surrounding bone surfaces but also seemed to be present at a low level in the DF. We confirmed these findings *in vitro* by identifying PTHrP mRNA at high levels in the cultured epithelial cells by RNase protection assay. The concentration of PTHrP secreted into the medium in 24 h was found to be ≈500 pM by a two-site IRMA assay. The expression of type I receptor mRNA in DF cells was also confirmed by RNase protection assay (Fig. 1). The modest PTHrP expression in the DF cell samples is presumably caused by the presence of contaminating SR cells as evidenced by staining of isolated clusters of cells with K14 in the early passages of DF cultures (data not shown). The functionality of the receptor was verified by treatment of DF cells with 10 nM PTHrP (1–40) (Sigma) for 10 min and documenting a greater than 5-fold induction in cAMP content in cell extracts.

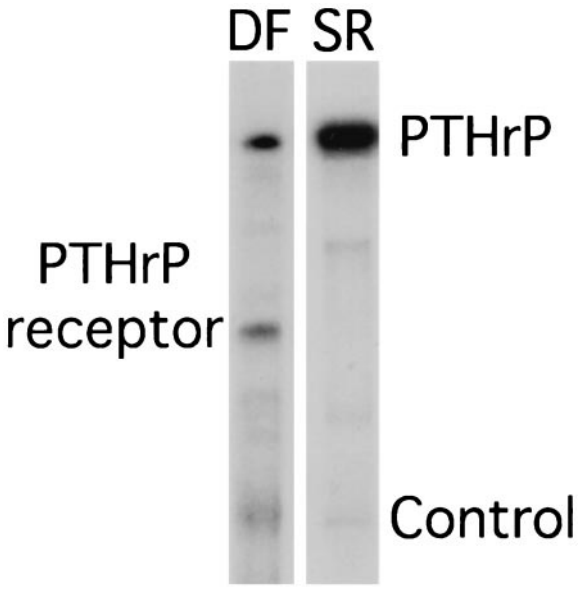

Fig. 1. (*A*) RNase protection assay for PTHrP and PTHrP receptor expression. Cultured SR cells express high levels of PTHrP without any expression of receptor. DF cells express the type I PTH/PTHrP receptor. The presence of PTHrP expression in the DF cells is presumably caused by contaminating SR cells (see text).

Osteoclasts Form Spontaneously in Cocultures of Dental Crypt Cells. Cocultures of SR and DF cells showed spontaneous osteoclast formation in the absence of added osteoclast precursors such as spleen or bone marrow suspensions and without induction with exogenous PTHrP or vitamin 1,25 $(OH)_2$ D. This result suggested that the primary cultures contain osteoclast precursors capable of forming osteoclasts in the presence of locally produced inducing substances. Osteoclasts were identified by their characteristic shape, the expression of three different markers, and testing for functionality with the pit formation assay. The first marker was TRAcP. TRAcP stains a tyrosine phosphatase expressed in osteoclast precursors and not in other multinucleated giant cells such as the macrophage polykaryons (Fig. 2*A*; refs. 16 and 24). The second was $\alpha_V\beta_3$ integrin (the vitronectin receptor), which is highly expressed in the osteoclasts and is needed for formation of tight attachments between the osteoclasts and the RGD-containing proteins in the bone. Its inhibition results in failure of bone resorption (Fig. 2*B*; ref. 17). The last was the presence of c-*src* (Fig. 2*C*), a proto-oncogene encoding an intracellular tyrosine kinase that is highly expressed in osteoclasts (18, 19, 25).

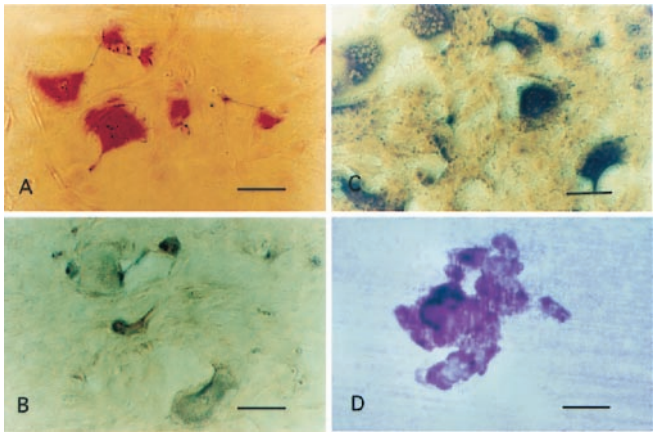

Fig. 2. Characterization of osteoclasts formed spontaneously in dental crypt cell cultures. (*A*) Staining for TRAcP. (*B*) Staining for $\alpha_V\beta_3$ integrin. (*C*) Staining for c-*src*. (*D*) Formation of pits on dentine slices by the osteoclasts. (Bars = 100 μm.)

We cultured dental crypt cells on dentine slices for 15 days followed by staining with toluidine blue to visualize pits and found that the spontaneously formed osteoclasts were fully functional as determined by their ability to form pits in dentine slices (Fig. 2*D*). The osteoclasts were seen *in vitro* as early as 3 days and as late as 16 days, with a peak at day 15 (Fig. 3).

PTHrP Is Required for Osteoclast Formation. We tested whether PTHrP is required for osteoclast formation in the primary cocultures by inhibiting endogenous PTHrP activity with a rabbit anti-human PTHrP (1–34) antiserum that had been successfully used in experiments of similar design (26). As measured by a 1–74 IRMA, PTHrP levels in conditioned media from primary cocultures of DF and SR were found to peak at a concentration of 100 pM at day 14. Using the rat osteosarcoma bioassay, we determined the amount of neutralizing antibody capable of completely inhibiting 1 nM (a 10-fold excess) of freshly added PTHrP and found this amount to be a 1:100 dilution of the antiserum. Treatment with this concentration of the antiserum produced a significant reduction in the number of TRAcP-positive cells in culture at day 14 as compared with control wells (340 vs. 1,179 osteoclasts per well in the treated vs. control cultures, respectively; $P = 0.006$; Fig. 4). Thus, PTHrP seems to be required for osteoclast formation in this system.

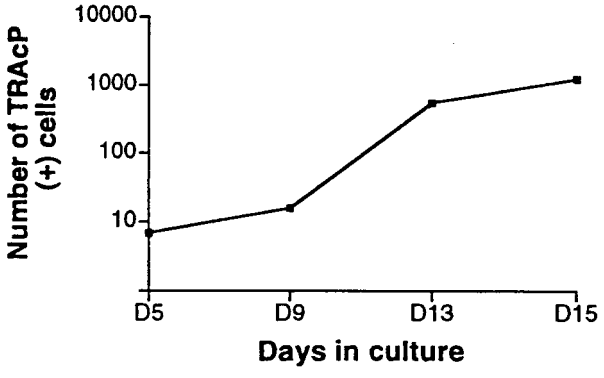

Fig. 3. Time course showing the number of TRAcP-positive mononucleated and multinucleated cells spontaneously formed in primary cultures of dental crypt cells. Each time point represents the average of three wells, and each well contained the cells obtained from one rat pup at postnatal day 8.

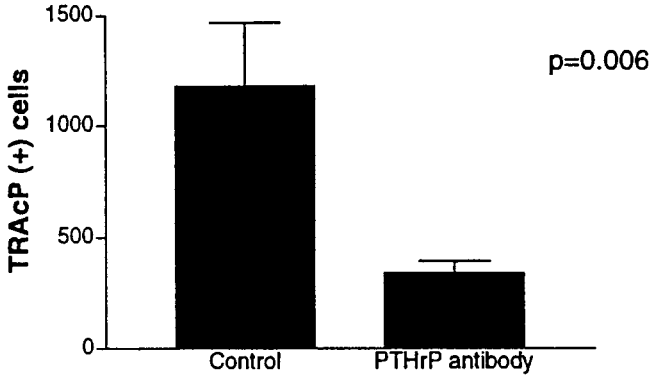

Fig. 4. The effect of addition of anti-PTHrP antibody on the number of osteoclasts formed by dental crypt cells. A rabbit anti-human PTHrP (1–34) antiserum was added daily to cultures of dental crypt cells. At day 15, the cultures were stained with TRAcP, and the average number of osteoclasts per well was determined. Control wells were treated with nonimmune rabbit serum. Bars represent means ± SEM ($n = 11$ for treated wells; $n = 9$ for control wells; $P = 0.006$).

DF Cells Mediate the Effects of PTHrP on Osteoclasts. Because bone-lining cells express the PTH/PTHrP type I receptor (6) and could potentially mediate osteoclast formation/differentiation, we first addressed the possibility that contamination of primary dental crypt cell cultures with this cell type might account for the spontaneous osteoclast formation. Because bone-lining cells express high levels of osteocalcin, we looked at osteocalcin expression *in vitro* as a marker for the presence of differentiated bone-lining cells. Although osteocalcin was expressed as early as 5 days *in vitro* in dental crypt cell cultures, Northern blot analyses for osteocalcin mRNA showed that in two of five cultures no osteocalcin could be detected at day 15, despite the presence of osteoclast formation *in vitro* (data not shown). These findings suggest that osteoclast formation can take place in the absence of cells expressing osteocalcin, including osteocalcin-expressing bone-lining cells.

In the tooth microenvironment, the DF is a likely target cell for the actions of PTHrP, as evidenced by the expression of the type I PTH/PTHrP receptor in these cells (see above). We therefore used the fetal-rat long-bone resorption assay to determine whether PTHrP induced DF cells to cause bone resorption. In four different experiments, conditioned media collected from PTHrP-treated DF cells stimulated ^{45}Ca release from labeled fetal bones by as much as 50% over that stimulated by control media (Fig. 5); the degree of stimulation was approximately equivalent to the stimulation achieved by adding 1 nM PTHrP to the bone resorption assay. Because PTHrP stimulates this assay directly, we controlled for the possible effects of both PTHrP carryover through the wash and PTHrP secretion from contaminating epithelial cells by assaying the collected conditioned media for PTHrP (1–34) by IRMA. We found PTHrP to be below the assay detection limit of 0.9 pM. These findings confirm that, in response to PTHrP, DF cells secrete a soluble molecule capable of inducing bone resorption (27).

The Role of ODF and OCIF. In the skeleton, osteoclast formation is induced by ODF on the cell surface of osteoblasts, and inhibited by soluble OCIF (23, 28). We performed Northern analyses for ODF and OCIF expression to determine whether these molecules are involved in osteoclast formation in the tooth microenvironment. Primary cocultures of dental crypt cells expressed both ODF and OCIF mRNA by day 15 of culture. We then focused on the DF cells, the presumed target cells in our system, by culturing them separately and treating them with 10 nM

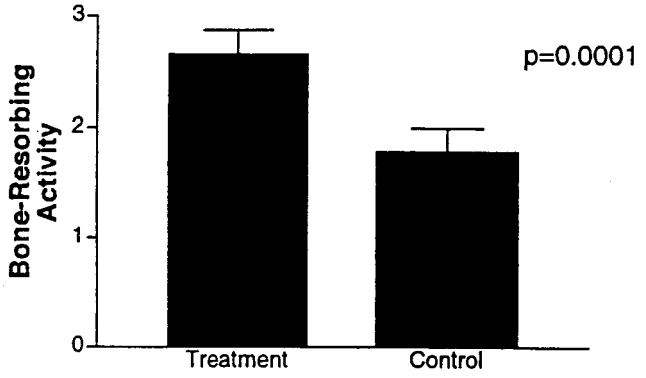

Fig. 5. Bone-resorbing activity of conditioned media from DF cells with and without PTHrP treatment. The *y* axis represents the fold induction over baseline resorption in conditioned media from DF cells treated or not treated with PTHrP (see *Materials and Methods*). Conditioned media were tested in four different experiments. Bars represent means ± SEM ($P = 0.0001$).

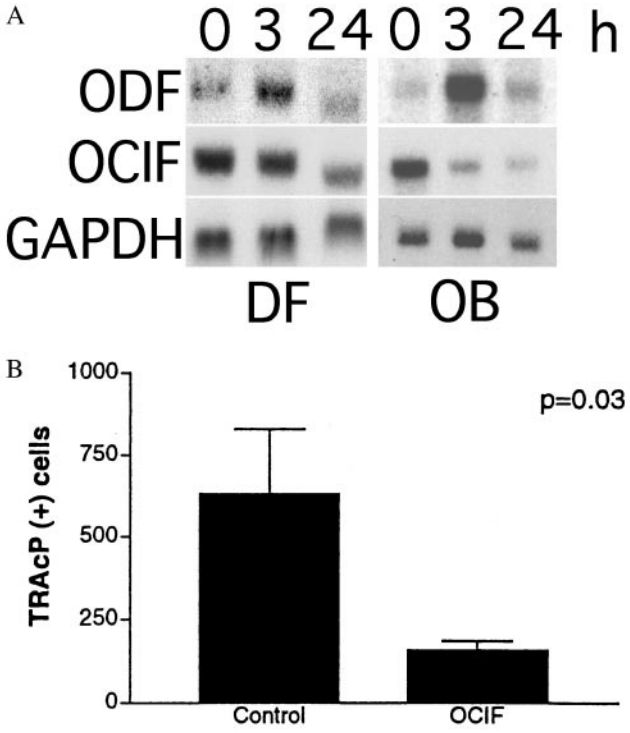

Fig. 6. (*A*) Northern blot analysis for ODF and OCIF in DF cells after the fourth passage and osteoblasts (OB) at baseline and at 3 and 24 h after treatment with 10 nM PTHrP (1–40). ODF was up-regulated by 3 h and rapidly returned to baseline. OCIF was down-regulated in both DF cells and osteoblasts by 24 h after treatment with PTHrP. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (*B*) The effect of addition of OCIF to cultures of dental crypt cells on the number of osteoclasts formed spontaneously. OCIF was added daily to primary cultures. At day 15, the cultures were stained with TRAcP, and the average number of osteoclasts per well was determined. Control wells were treated with mouse IgG. Bars represent means ± SEM ($n = 8$ for treated and control wells; $P = 0.03$).

PTHrP (1–40). Northern analyses were performed on RNA obtained at time 0 as well as 3 and 24 h after addition of PTHrP and compared with analyses of RNA from similarly treated osteoblasts. We found that ODF was up-regulated in DF cells at 3 h but returned to baseline at 24 h, whereas OCIF was down-regulated at 24 h. This time course is qualitatively similar to the effect of 10 nM PTHrP treatment on osteoblasts (Fig. 6*A*) and has been reported in stromal cell lines able to support osteoclast formation (29). We next attempted to block ODF signaling in our cocultures by the daily addition of OCIF and found a significant decrease in the number of TRAcP-positive cells at day 15 (158 vs. 631 osteoclasts per well in the treatment vs. control cultures, respectively; $P = 0.03$; Fig. 6*B*).

Discussion

Gene manipulation experiments have established that PTHrP action is required for tooth eruption (6). In normal mice, the peak in PTHrP expression in the SR overlying the first molar at day 10 corresponds to the onset of maximal osteoclastic resorption, suggesting that PTHrP may be a critical signal in the regulation of osteoclastic differentiation/maturation as PTH does elsewhere in the skeleton (30). In the rescued PTHrP-knockout mouse, which is PTHrP null at all sites except cartilage, progressive impaction and distortion of the teeth were observed. Although it was our initial impression that the number of osteoclasts in the dental crypt was normal in these mice, the degree of impaction of the crypts made an accurate assessment of osteoclast number impossible. Because the long bones of PTHrP-knockout mice are not osteopetrotic (5), bone resorption in these sites does not seem to be impaired. Thus, our findings clearly indicated that the defect in osteoclast regulation in these mice was specific to the microenvironment of the tooth. The N-terminal homology between PTH and PTHrP as well as their shared receptor suggest that if PTHrP plays a role in bone resorption, it should be through the same signaling cascade used by PTH. Two conclusions would follow from this assumption. First, one would expect an effect on osteoclast formation as well as activity (15). Second, because osteoclasts do not seem to bear a functional type I PTH/PTHrP receptor, an intermediary cell and signal should be involved (the osteoblast in the case of PTH itself; ref. 10).

The progressive increase in the number of osteoclasts in our cultures to a peak at 15 days indicates that these cells were forming *de novo* and not accidentally carried over from the alveolar crypt, because the life span of an osteoclast *in vitro* rarely exceeds 10 days. The spontaneous formation of osteoclasts in these cultures was unexpected, because the induction of osteoclasts *in vitro* usually requires the addition of a great excess of stromal cells able to support osteoclast formation and supraphysiologic doses of an inducer such as vitamin 1,25 $(OH)_2$ D or PTH. Thus, it is clear that the cell types within the dental crypt are capable of providing the signals required to support osteoclast formation.

We used an RNase protection assay to confirm that the SR cells in the cultures express PTHrP, as previously suggested by *in situ* hybridization (6). Endogenous PTHrP is clearly required for the formation of crypt osteoclasts in our culture system, because the addition of PTHrP antiserum significantly decreased the number of osteoclasts formed *in vitro* as compared with that in the control serum. In addition, our findings indicated that osteoclast formation could proceed in the dental crypt cultures in the apparent absence of osteoblasts, thus implicating another stromal cell as a mediator. DF cells were logical candidates, because dissection experiments by Larson *et al.* (31) had revealed that tooth eruption does not proceed in their absence. We confirmed by RNase protection analysis that DF cells express the type I PTH/PTHrP receptor. The receptor is fully functional, and its response to PTHrP as assayed by the stimulation of adenylate cyclase is equivalent to that of mammary mesenchymal cells, a known target for PTHrP action (32). These findings suggest that PTHrP expressed by epithelial cells surrounding the tooth acts via a paracrine cascade on the DF cells to induce osteoclast formation/activation, which results in the formation of an eruption pathway. This hypothesis is sup-

ported by our observation that PTHrP treatment of DF cells resulted in the induction of a soluble mediator that increased bone resorption in the fetal-rat long-bone assay. This mediator is as yet unidentified and probably is unrelated to ODF.

We were also able to demonstrate that the ODF/OCIF system critical to osteoblast-mediated stimulation of osteoclast formation in the peripheral skeleton seems to exhibit an analogous expression pattern in the dental crypt (23). In particular, we found an increase in ODF expression on stimulation of DF cells with 10 nM PTHrP, with a time course resembling that observed in osteoblasts treated with PTHrP (although the increase was less pronounced). We further observed down-regulation of OCIF expression with PTHrP treatment, again similar to the effects seen in osteoblasts. These findings support the notion that the DF cells replace the osteoblasts in the tooth microenvironment. The molecular mechanisms involved in osteoclast regulation thus seem similar in the skeleton and the tooth microenvironment.

DF cells express colony-stimulating factor-1 (CSF-1), with a peak at day 3 in the first molar in rodents, coinciding with maximal monocyte influx in the microenvironment of the tooth (33). CSF-1 is required not only for recruitment of monocytes but is also known to be important for the proliferation of osteoclast progenitors and survival of osteoclasts (34, 35). PTHrP, on the other hand, is expressed later. Its expression increases gradually as CSF-1 expression diminishes, with a peak at postnatal day 10, coinciding with the formation of an eruption pathway over the crown of the tooth (6). The late expression of PTHrP as compared with that of CSF-1 at a time of active uncoupled osteoclastic bone resorption suggests that PTHrP is required for the later steps of formation/differentiation/activation of osteoclasts in the tooth microenvironment. In our culture system, it is known that CSF-1 is constitutively expressed in the DF, and we have proven that osteoclast formation does not proceed in the absence of PTHrP (33, 36). Thus, CSF-1 presumably is the permissive factor for osteoclast formation required before PTHrP completes the formation of osteoclasts. Further study is required to elucidate the exact role of PTHrP in osteoclast development and regulation in this system.

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