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Differential Effects of Intermittent and Continuous Administration of Parathyroid Hormone on Bone Histomorphometry and Gene Expression

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A mechanism explaining the differential skeletal effects of intermittent and continuous elevation of serum parathyroid hormone (PTH) remains elusive. Intermittent PTH increases bone formation and bone mass and is being investigated as a therapy for osteoporosis. By contrast, chronic hyperparathyroidism results in the metabolic bone disease osteitis fibrosa characterized by osteomalacia, focal bone resorption, and peritrabecular bone marrow fibrosis. Intermittent and continuous PTH have similar effects on the number of osteoblasts and bone-forming activity. Many of the beneficial as well as detrimental effects of the hormone appear to be mediated by osteoblast-derived growth factors. This hypothesis was tested using cDNA microgene arrays to compare gene expression in tibia of rats treated with continuous and pulsatile administration of PTH. These treatments result in differential expression of many genes, including growth factors. One of the genes whose steady-state mRNA levels was increased by continuous but not pulsatile administration was platelet-derived growth factor-A (PDGF-A). Administration of a PDGF-A antagonist greatly reduced bone resorption, osteomalacia, and bone marrow fibrosis in a rat model for hyperparathyroidism, suggesting that PDGF-A is a causative agent for this disease. These findings suggest that profiling changes in gene expression can help identify the metabolic pathways responsible for the skeletal responses to the hormone.

Key Words: Parathyroid hormone; rat bone; bone histomorphometry; platelet-derived growth factor.

Introduction

The contrasting skeletal responses caused by parathyroid hormone (PTH)—anabolic for intermittent (pulsatile) and detrimental for chronic hyperparathyroidism—have been described at the histologic level *(1–8)*. The skeletal abnormalities caused by hyperparathyroidism have been known for more than 70 yr. In spite of intense interest in PTH as a treatment to cure established osteoporosis *(9)* and the clinical importance of hyperparathyroidism *(4,5)*, the biochemical and molecular mechanisms that mediate the differential response of the skeleton to continuous and intermittent increases in circulating PTH have resisted characterization and are essentially unknown. This, in part, may have been owing to the lack of in vitro models that replicate the skeletal abnormalities. Excellent animal models for intermittent PTH, hyperparathyroidism, and renal osteodystrophy (a metabolic bone disease caused by secondary hyperparathyroidism) have been available for many years, but the perceived complexity of these in vivo models appears to have discouraged investigators from pursuing mechanistic studies. However, a close examination of the histologic changes in bone following intermittent and continuous PTH treatment in animals suggests a unifying model of PTH action. In this article, this model and recent studies directed toward identifying the molecular basis for the bone anabolic response to intermittent PTH as well as skeletal abnormalities caused by hyperparathyroidism are discussed.

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Continuous and Pulsatile Administration of PTH Have Similar Effects on Bone Formation

The changes in bone histology induced by continuous and intermittent PTH have been most completely described in rats. Intermittent sc administration of PTH increases bone mass in growing and adult rats as a result of an increase in bone formation *(1–3,10–16)*. The increase in bone formation is owing primarily to an increase in osteoblast number; increases in osteoblast activity make a much smaller contribution to the overall response. The PTH-induced increase in bone formation takes place on previously quiescent bone surfaces *(17)*. Thus, by definition, the increase in bone formation reflects an increase in bone modeling, not bone remodeling. This is an important distinction because were PTH to act by increasing bone remodeling, bone resorption would have preceded the increase in bone formation *(18,19)*.

A continuous sc infusion of ^{3}H-thymidine was given to label all cells progressing through the S phase of the cell cycle of rats during treatment with PTH. No increase in labeling of osteoblasts was observed, indicating that enhanced

proliferation of osteoprogenitor cells contributes little to the initial increase in osteoblast number *(20)* (Fig. 1). Similar results were obtained following administration of slow-release bromodeoxyuridine *(21)*. The results of these two experiments indicate that PTH is capable of rapidly increasing osteoblast number by acting on existing cells. Subsequent studies suggest that the anabolic skeletal response to PTH in humans is similarly rapid *(22)*.

Apoptosis is another method of regulating cell number. Apoptosis is unlikely to play a role in the initial increase in bone formation in response to PTH treatment. Apoptosis of osteoblasts was not decreased in short-term studies, as would be required to increase cell number *(23)*. Additionally, the increase in osteoblast number in response to PTH (up to 1000%) occurs within 1 wk. The average life span of cells of the osteoblast lineage (osteoblasts, bone-lining cells, and osteocytes) is much longer *(24)*. As a consequence, an increase in life span would have a minimal influence on cell number during this interval. However, it is possible that changes in osteoblast recruitment and apoptosis could contribute to the continued maintenance of bone formation during long-term treatment with PTH *(25)*.

The most likely target cell for PTH in bone is the bone-lining cell. These cells are derived from osteoblasts and are identified by location (immediately adjacent to bone surfaces) and morphology (thin mononucleated cells). Lining cells are thought to be inactive osteoblasts. The classic morphologic description of an osteoblast is a large, cuboidal cell located immediately adjacent to a bone surface and having an asymmetrically positioned nucleus and clear zone (corresponding to the Golgi apparatus). Osteoblasts usually present themselves histologically as a monolayer array of closely associated cells. When visualizing a bone-forming surface, it is obvious that there are many cells expressing phenotypes intermediate between osteoblasts and bone-lining cells *(24)*. Observation of fluorochrome labeling suggests that the rate of matrix synthesis by cells of the lining cell/osteoblast lineage is positively related to cell size, but quantitative measurements have not been performed to confirm this impression *(24)*.

Leaffer et al. *(26)* proposed that intermittent PTH treatment increases osteoblast number by activating the bone-lining cells. Dobnig and Turner *(20)* came to the same conclusion and termed the process *modulation*, whereby the osteoblast and lining cell phenotypes can be interconverted. PTH *(20,26)* and mechanical loading *(27)* appear to increase osteoblast number on cancellous bone surfaces by modulating the lining cell phenotype to an osteoblast phenotype, whereas estrogen decreases osteoblast number on calvarial periosteum by modulating the osteoblast to a lining-cell phenotype *(28)*.

Circumstantial evidence strongly suggests that many PTH-induced osteoblasts are derived via modulation of the quiescent (in terms of bone formation) lining cells to the osteoblast phenotype, but it is possible that other cells located within the osteogenic layer have this capacity as well. PTH increases bone formation on cortical bone surfaces with a delayed response compared to cancellous bone, suggesting an alternative mechanism of action. However, the origin of the PTH-induced osteoblasts on cortical bone surfaces has not been investigated.

Some, but not all, studies report increased bone formation following continuous infusion of PTH into rats *(3, 6–8,29)*. Increased bone formation has also been reported in patients with hyperparathyroidism.

We recently reviewed a series of 605 charts from patients diagnosed with hyperparathyroidism who had iliac crest bone biopsies measured in our histomorphometry core facility, Mayo Clinic, Rochester, MN. A striking 90% of these patients had bone marrow fibrosis. Other common abnormalities included osteomalacia, increased bone resorption, and increased bone formation. Cancellous osteopenia was infrequently observed, but cortical thinning, although not quantified, was frequently observed. Findings similar to these have been reported in smaller series of patients with hyperparathyroidism *(30,31)*.

An increase in bone formation was observed in approx 50% of the biopsies from patients diagnosed with hyperparathyroidism (Fig. 2). However, in reviewing human bone biopsies, we noticed that bone formation was not measured in almost half of the biopsies from patients with hyperparathyroidism because the fluorochrome labels were too diffuse to distinguish double labels. We observed a similar phenomenon in some of the histologic sections from our rat studies in which the animals were infused continuously with PTH *(29)*. Rapid bone matrix deposition with delayed mineralization produces diffuse fluorochrome labeling. Thus, it is likely that the bone matrix synthesis in patients with hyperparathyroidism and rats treated with continuous PTH has often been underestimated.

We have recently reexamined the effects of continuous PTH on bone formation in the rat. After 1 wk of treatment, bone formation was increased to an equal extent by continuous and intermittent PTH (unpublished data). Both methods of administering PTH also resulted in nearly identical increases in steady-state mRNA levels for type I collagen, osteocalcin, and osteonectin (Fig. 3). Thus, continuous and intermittent administration of PTH appear to have similar effects on osteoblast number, activity, and matrix synthesis. However, there is clearly a difference in mineralization rate between intermittent administration of PTH and hyperparathyroidism, with the latter demonstrating a high incidence of delayed mineralization.

Skeletal Abnormalities Associated with Hyperparathyroidism

The symptomatology of the development of osteitis fibrosa in chronic hyperparathyroidism depends on the severity and duration of the disease, and includes in increasing severity

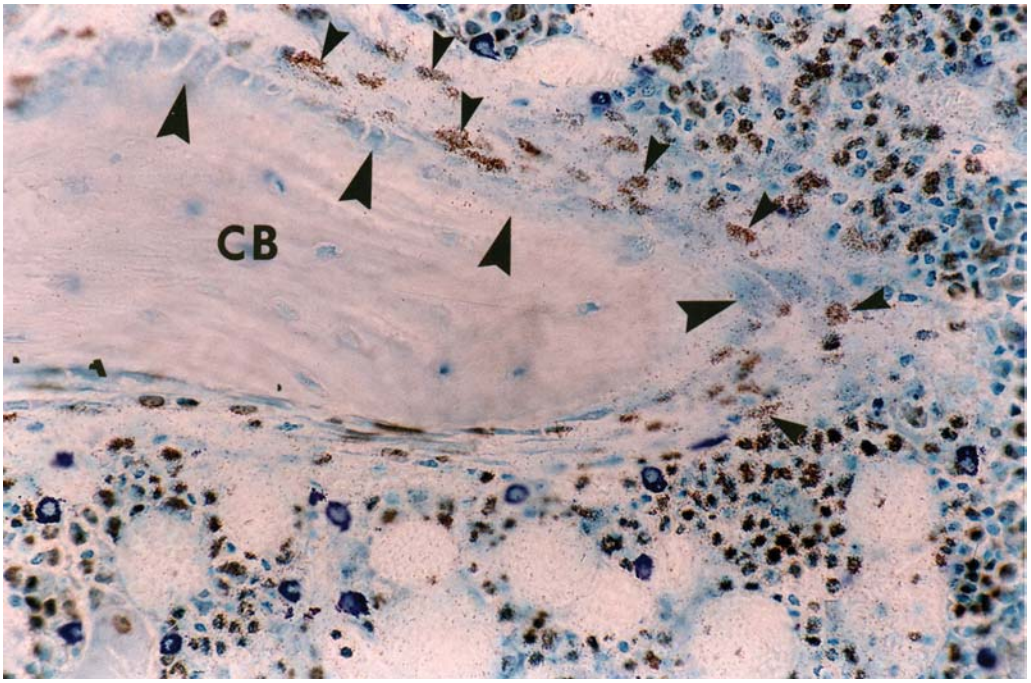

Fig. 1. Radioautograph of rat treated with continuous administration of human PTH (1–34) (40 μg/[kg·d]) for 1 wk. ^{3}H-thymidine was delivered continuously, using an sc implanted osmotic pump, to label all cells progressing through S phase of the cell cycle. Note that the osteoblasts (large arrow heads) located immediately adjacent to cancellous bone (CB) are unlabeled, whereas many of the peritrabecular fibroblasts (small arrowheads) are labeled. These results demonstrate that the PTH-induced osteoblasts originate from existing cells, whereas many of the fibroblasts originate from proliferation.

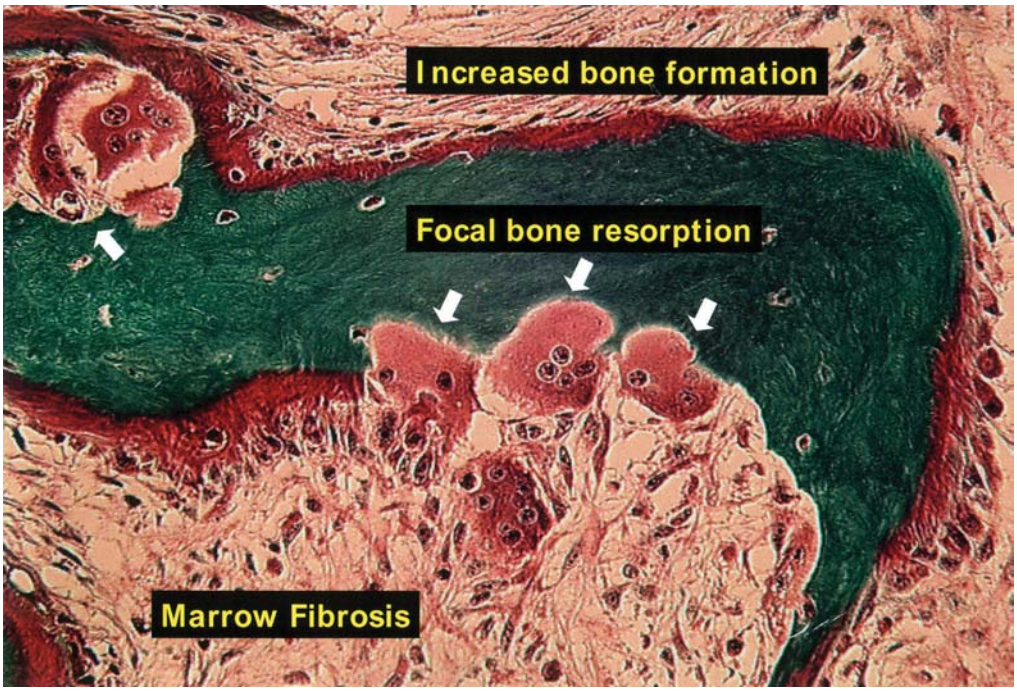

Fig. 2. Histologic presentation of osteitis fibrosa in patient with hyperparathyroidism. Excess osteoid (bright red–stained tissue adjacent to green-stained mineralized bone matrix), focal bone resorption (arrows), increased bone formation, and extensive bone marrow fibrosis are readily apparent. Note the proximity of fibroblasts to cancellous bone (peritrabecular). The histologic presentation of osteitis fibrosa in patients with hyperparathyroidism and rats treated continuously with PTH (Fig. 1) suggests that PTH target cells on bone surfaces (e.g., lining cells/osteoblasts) produce growth factors that attract fibroblasts and stimulate their proliferation.

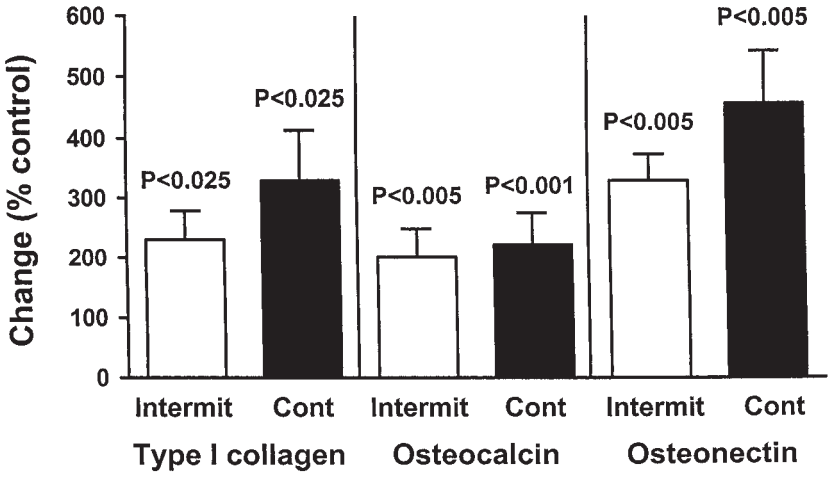

Fig. 3. Intermittent (Intermit) and continuous (Cont) administration of PTH result in similar increases in steady-state mRNA levels for bone matrix proteins. Human PTH (1–34) (80 μg/[kg·d]) was administered daily by the sc route (Intermit) or by an sc implanted osmotic pump (40 μg/[kg·d]) (Cont) for 1 wk. Total cellular RNA was isolated from the proximal tibial metaphysis, and mRNA levels were measured by Northern blot analysis. The values were corrected for differences in loading by normalizing them to 18S ribosomal RNA and the data expressed as percentage of carrier-treated control. The values are expressed as the mean ± SEM ($n = 4$), and the p values refer to significance compared to the controls. No significant differences were observed between intermittent and continuous PTH.

(1) tunneling of trabeculae by osteoclasts and excess osteoid formation by osteoblasts; (2) bone resorption accompanied by fibrosis around the weakened trabeculae; and (3) replacement of marrow by fibrous tissue, hemorrhage from microfractures, and hemosiderin-laden macrophages that often display osteoclast-like giant cells resulting in a cystic brown tumor *(33)*.

Continuous infusion of PTH in rats (Table 1) for 1 wk or longer results in skeletal pathology strikingly similar to that of patients with hyperparathyroidism *(28)*. The abnormalities include extensive peritrabecular fibrosis, osteomalacia, increased bone formation, and focal bone resorption. Thus, the rat model for hyperparathyroidism appears to replicate the human metabolic bone disease with a high degree of fidelity.

The relationship between skeletal abnormalities and the duration of a PTH pulse has been more precisely defined by programming osmotic pumps to deliver the same quantity of PTH over different intervals (Fig. 4) *(29)*. A 1-h pulse induced a skeletal response similar to daily (intermittent) sc administration. By contrast, detrimental side effects were observed following administration of PTH using daily pulses as short as 2 h, and these detrimental effects increased with pulse duration to have the same effect as continuous PTH with pulses lasting 6 h (Fig. 5). These results suggest that as long as a minimum effective dose is obtained, the duration of exposure to PTH is a more important variable than peak serum levels of the hormone. Furthermore, the maximum duration of a PTH pulse required to increase bone formation without having detrimental side effects is no more than 1 h. Finally, there appears to be a continuum of action of PTH to induce bone formation and abnormalities that depends on the precise duration of exposure to the hormone.

Table 1
Comparative Effects of Intermittent and Continuous PTH on Bone Histomorphometry in Rats[a]

Measurement	Intermittent PTH	Continuous PTH
Osteoblast perimeter	⇑*	⇑*
Bone formation rate	⇑*	⇑*
mRNA levels for bone matrix proteins	⇑*	⇑*
Osteoclast perimeter	⇔*	⇑[b]
Marrow fibrosis	Not observed*	Extensive[†]
Osteomalacia	Not observed*	Extensive[†]

[a] Measurements were performed at the proximal tibial diaphysis. ⇑, increased compared to untreated control; ⇔, no change. The same superscript symbol means there were no differences between intermittent and continuous PTH; different superscript symbols mean intermittent and continuous PTH differ; $p < 0.05$. See ref. *30* for details.

^{3}H-thymidine radioautography was performed to determine the role of cell proliferation in contributing to the increases in osteoblasts and fibroblasts following continuous administration of PTH. ^{3}H-thymidine was infused continuously for the entire 1-wk duration of PTH treatment in order to label all proliferating cells. As was the case in earlier studies investigating intermittent administration of PTH, the osteoblasts induced by continuous administration of PTH were unlabeled, indicating that they were derived by modulation rather than proliferation. In contrast to osteoblasts,

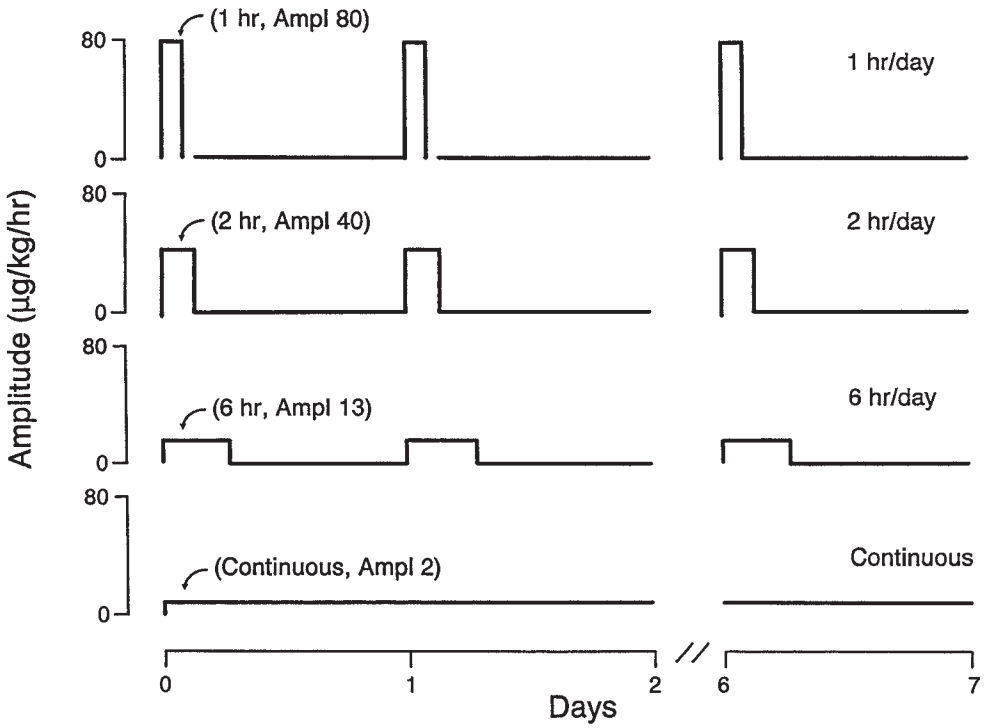

Fig. 4. Idealized pattern of changes in serum PTH levels following programmed delivery of human PTH (1–34) (80 µg/[kg·d]) using sc implanted osmotic pumps. The area under the curve is identical for each treatment (except continuous), but the peak amplitude of serum PTH decreases as the duration of the pulse delivered by the pump is increased. The dose rate for continuous PTH was reduced to 40 µg/(kg·d) because the higher dose rate resulted in life-threatening hypercalcemia.

Side effect	Programmed delivery				
	SC	*1 hr*	*2 hr*	*6 hr*	*Cont*
Weight loss	O	O	+	+++	+++
Hypercalcemia	O	O	O	+++	+++
Hyperphosphatemia	+	O	O	O	O
Fibrotic marrow	O	O	O	+++	+++

Fig. 5. Side effects associated with increasing duration of exposure to PTH. SC, subcutaneous; O, no side effect observed. The number of plus signs indicates the relative severity of the side effect, with + as least severe and +++ as most severe. See ref. *30* for experimental details.

most of the peritrabecular fibroblasts induced by continuous infusion with PTH were labeled with ^{3}H-thymidine, indicating that these cells had progressed through the cell cycle. Thus, PTH-induced osteoblasts and fibroblasts originate by different cellular pathways.

The critical role of osteoblast-derived autocrine and paracrine factors in the regulation of normal bone turnover is well recognized *(8)*. The close association of fibroblasts with bone surfaces in osteitis fibrosa, the important role of fibroblast cell proliferation in that disease, and the apparent absence of an effect of PTH on cultured bone marrow fibroblasts suggest that continuous administration of PTH results in the release of paracrine factors that are chemotactic to fibroblasts and stimulate their proliferation (Fig. 6). Similarly, we postulate that the osteomalacia and focal bone resorption induced by hyperparathyroidism may also be owing to overproduction of osteoblast-derived factors.

Evidence for Differential Regulation of Gene Expression by Intermittent and Continuous Administration of PTH

As previously discussed, intermittent and continuous administration of PTH have similar effects on bone matrix

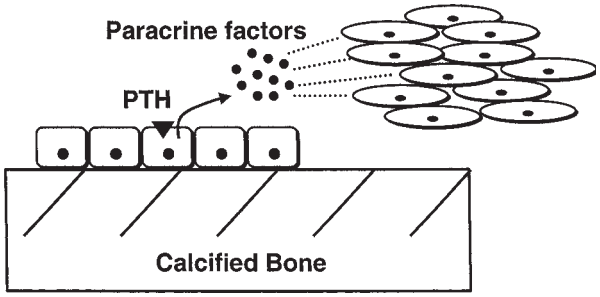

Fig. 6. Model for hyperparathyroidism-induced osteitis fibrosa. PTH acts via its receptor on bone-lining cells to modulate them to express the osteoblast phenotype and produce bone matrix. Autocrine and paracrine growth factors, which play a critical role in the induction and organization of the osteogenic layer required for renewed bone formation, are produced by these cells in response to PTH. Overexpression of some of these factors attracts fibroblasts and induces their proliferation, leading to peritrabecular bone marrow fibrosis. Osteoblast and/or fibroblast-derived growth factors are also responsible for osteomalacia and focal bone resorption, which accompany hyperparathyroidism.

gene expression in rats. However, recent studies suggest that the two methods of administering PTH result in major differences in regulation of expression of many other genes. Total cellular RNA was isolated from the proximal tibial metaphysis of rats treated with intermittent and pulsatile PTH *(33)*. cDNA microarray analysis was performed using this RNA to identify differentially expressed genes in bone. Animals treated with pulsatile PTH were compared with controls as well as with rats given continuous PTH in order to identify candidate genes that mediate the detrimental effects of continuous PTH. At a confidence level of $p < 0.05$, gene expression analysis of 8500 rat genes demonstrated that 3.6% of the genes were regulated by intermittent administration of PTH and 10.4% by continuous administration of PTH. Of the regulated genes, 158 were unique to intermittent administration of PTH and 759 to continuous administration of PTH. An additional 158 regulated genes were common to both treatments. Classification of regulated genes by pathways and function identified similarities and differences. Protein cleavage and degradation was the pathway most represented in genes unique to continuous administration of PTH and included components of the ubiquitin-proteosome degradation complex, as well as metalloproteases and their inhibitors. Based on functional classification, genes uniquely regulated by continuous administration of PTH encoded many 7-transmembrane/G protein–coupled receptors, and integral membrane proteins. Genes common to both intermittent and continuous administration of PTH included many integral membrane proteins and extracellular matrix proteins. These results suggest that the anabolic and detrimental effects of PTH on bone regulated both common and unique subsets of genes and pathways.

The cause-and-effect relationship between the gene changes and morphologic changes in bone in response to PTH is of great interest because it may reveal targets for novel therapies to prevent and cure hyperparathyroidism-induced bone disease. The gene array data suggest that intermittent and continuous administration of PTH uniquely regulate the expression of hundreds if not thousands of genes in bone tissue. It is not practical to evaluate the cause-and-effect relationship for such large numbers of genes. However, the model shown in Fig. 6 suggests that the beneficial as well as detrimental skeletal effects of PTH are attributable to growth factors. We screened the long list of regulated genes for growth factors and their receptors and observed regulation of several dozen genes by PTH.

Our initial studies to demonstrate cause-and-effect relationships have focused on osteitis fibrosa. Based on the morphologic changes induced by hyperparathyroidism, we anticipated that the causative agent would be a growth factor that is produced by osteoblasts, is chemotactic to fibroblasts, stimulates fibroblast proliferation, induces bone resorption, and is upregulated by continuous but not intermittent administration of PTH. Platelet-derived growth factor-A (PDGF-A) meets all of these criteria *(34)*.

PDGF is the major growth factor in blood. It is synthesized by megakaryocytes and is transported in blood stored in the α granules of platelets. PDGF is a homo- or heterodimer of two polypeptide chains, PDGF-1 (PDGF-A) and PDGF-2 (PDGF-B), which show 56% homology and are linked by disulfide bonds. The PDGF-A chain is encoded by a gene located in chromosome 7, and PDGF-B chain is encoded by the c-*sis* protooncogene localized in chromosome 22 *(35)*. The PDGF heterodimer and its two isoforms, the PDGF-A and PDGF-B homodimers, are potent mitogenes and chemoattractants for target cells such as fibroblasts and osteoblasts *(36–45)*. PDGF has also been shown to increase bone resorption in vitro *(36,37)*. There are two PDGF receptors. The PDGF-A homodimer binds only to its specific receptor (α), and the PDGF heterodimer and PDGF-B homodimer bind to both receptors (α and β) *(35)*.

In skeletal tissues, PDGF-A gene is expressed in normal osteoblasts, malignant skeletal cells, and osteosarcoma cell lines *(36–45)*. The prevalence of the PDGF-A receptor on osteoblasts but not on osteoclasts at remodeling sites suggests that the osteoblast is a target of PDGF-A. The production of PDGF-A by osteoblasts suggests an autocrine and/or paracrine role for PDGF-A in the regulation of osteogenesis, possibly in the spatial organization of cells in the osteogenic compartment *(37–45)*.

PDGF is believed to play a role in the genesis of pathologic fibrosis, including marrow fibrosis in patients with myeloproliferative diseases *(46–54)*. The growth factor has been implicated in the pathogenesis of liver fibrosis, a major determinant of the clinical course of chronic liver disease *(47)*. Idiopathic pulmonary fibrosis is characterized by alveolar macrophages that spontaneously release exaggerated

amounts of PDGF, suggesting that elevated PDGF plays a causative role in this condition *(48)*.

PDGF-A action is inhibited by triazolopyrimidine (trapidil). Trapidil, originally developed as a vasodilator and antiplatelet agent, has proven to be clinically effective in the treatment of coronary heart disease *(55–62)*. Although the mechanism of its action has not been fully deciphered, there is evidence that trapidil inhibits PDGF signaling via competitive binding to PDGF receptor *(60)*.

The effects of trapidil on bone histology were investigated in a rat model for chronic hyperparathyroidism *(34)*. Trapidil alone had no effect on serum chemistry or bone histomorphometry. As expected, continuous sc infusion of PTH for 1 wk increased serum PTH, serum calcium, osteoblast number, osteoclast number, and bone formation and induced extensive osteomalacia and peritrabecular fibrosis. Trapidil had no significant effects on PTH-induced increases in serum PTH, osteoblast number, and bone formation. However, trapidil decreased hypercalcemia, peritrabecular fibrosis, and osteoclast perimeter. These results support the hypothesis that excess PDGF-A is essential for PTH-induced bone marrow fibrosis and focal bone resorption and that drugs that target PDGF-A signaling can reduce or prevent the development of skeletal pathologies induced by hyperparathyroidism.

Although PDGF-A signaling appears to be a contributing factor to the development of PTH-induced osteitis fibrosa, other growth factors are likely to be involved as well. For example, differential regulation of the expression of members of the RANK, RANK ligand, osteoprotegerin system, which plays a critical role in osteoclast differentiation by PTH, is likely to play an important role in focal bone resorption induced by hyperparathyroidism *(63–65)*. Other studies suggest that overproduction of stem cell factor by osteoblasts might be responsible for accumulation of mast cells in the bones of patients with hyperparathyroidism *(66)*.

Conclusion

PTH is a potent stimulator of bone formation, and under some circumstances, treatment with PTH can increase bone formation and restore bone to an osteopenic skeleton. Continuous and intermittent administration of PTH increase osteoblast number and activity to a similar extent. However, as the duration of exposure to increased PTH levels is increased, the severity of side effects increases. These side effects include osteomalacia, focal bone resorption, and peritrabecular fibrosis. Recent studies suggest that analyses of cDNA microgene arrays provide a powerful tool that can be used to help identify and characterize the signaling pathways associated with the beneficial and detrimental side effects of PTH. This technique is insufficient, however, to establish cause-and-effect relationships. By combining histologic analysis, gene expression analysis, and the use of specific metabolic inhibitors, it may be possible to gain insight into the role of specific metabolic events in mediating the physiologic, pathologic, and pharmacologic actions of PTH.

Acknowledgments

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