Parathyroid bone disease in humans is caused by chronic hyperparathyroidism (HPT). Continuous infusion of PTH into rats results in histological changes similar to parathyroid bone disease, including increased bone formation, focal bone resorption, and severe peritrabecular fibrosis, whereas pulsatile PTH increases bone formation without skeletal abnormalities. Using a cDNA microarray with over 5000 genes, we identified an association between increased platelet-derived growth factor-A (PDGF-A) signaling and PTH-induced bone disease in rats. Verification of PDGF-A overexpression was accomplished with a ribonuclease protection assay. Using immunohistochemistry, PDGF-A peptide was localized to mast cells in PTH-treated rats. We also report a novel strategy for prevention of parathyroid bone disease using triazolopyrimidine (trapidil). Trapidil, an inhibitor of PDGF signaling, did not have any effect on indexes of bone turnover in normal rats. However, dramatic reductions in marrow fibrosis and bone resorption, but not bone formation, were observed in PTH-treated rats given trapidil. Also, trapidil antagonized the PTH-induced increases in mRNA levels for PDGF-A. These results suggest that PDGF signaling is important for the detrimental skeletal effects of HPT, and drugs that target the cytokine or its receptor might be useful in reducing or preventing parathyroid bone disease. (Endocrinology 144: 2000–2007, 2003)

**Materials and Methods**

**Animals**

Three-month-old female Sprague Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) were housed on a 12-h light, 12-h dark cycle with standard laboratory chow and water *ad libitum*. All procedures were approved by the Mayo Foundation institutional animal care and use committee.

Abbreviations: BW, Body weight; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPT, hyperparathyroidism; hPTH, human PTH-(1–34); PDGF-A, platelet-derived growth factor-A; RNase, ribonuclease.
Study 1: induction of parathyroid bone disease

This experiment was performed to identify candidate genes and localize target cells that play a role in the detrimental effects of continuous PTH on bone. Animals were randomly divided into three groups, with five animals per group. One group was given daily sc injection of 80 μg/kg body weight (BW) hPTH-(1–34) (hPTH) for 7 d. The two other groups received sc implanted osmotic pumps (Alza Corp., Mountain View, CA) that delivered vehicle or 40 μg/kg BW·d hPTH at the rate of 1 μl/h for 7 d. On d 8 animals were anesthetized with ketamine (50 mg/kg BW)/xylazine HCl (5 mg/kg BW) and killed by decapitation, and both tibiae and femora were removed. Right tibiae were fixed by immersion in 70% ethanol and processed for bone histo-logic to verify the appearance of peritrabecular fibrosis. Left tibiae were frozen in liquid N2 and stored frozen until processed for RNA isolation, cDNA microarray, and ribonuclease (RNase) protection assay. Femora were fixed in 10% neutral buffered formalin for immuno-histochemistry.

Study 2: effect of trapidil on parathyroid bone disease

This experiment was performed to evaluate the effect of trapidil, a PDGF antagonist, on bone in rats treated with continuous PTH. Rats were divided into four groups [vehicle (n = 9), trapidil (n = 10), PTH (n = 8), and PTH plus trapidil (n = 10)]. The animals were implanted sc for 1 wk with osmotic pumps containing either vehicle or 40 μg/kg BW·d hPTH. They also received daily sc injections of vehicle or 40 μg/kg BW·d trapidil (Rodleben Pharma GmbH, Rodleben, Germany) for 8 d. This dosage was estimated on the basis of an effective inhibitory effect of trapidil on several types of cells in the rat (12–14). Fluorochrome labels (20 mg/kg BW; Sigma-Aldrich, St. Louis, MO) were injected at the base of the tail on d 0 (tetracycline) and d 6 (calcein). At the end of experiment (d 8), the rats were anesthetized, and blood was collected by cardiac puncture for determination of serum chemistry and PTH levels before death. Tibiae were removed and fixed in 70% ethanol for bone histomorphometry. Femora were stored frozen at −80 C for RNA isolation and RNase protection assay.

Isolation of RNA

The frozen proximal tibial metaphyses and distal femoral metaphyses were individually homogenized in guanidine isothiocyanate using a Spex freezer mill (Spex Industries, Inc., Edison, NJ). Total RNA was extracted and isolated using a modified organic solvent method, and the RNA yields were determined spectrophotometrically at 260 nm (15).

cDNA microarray analysis

Rat gene filter microarrays (GF 300) consisting of 5531 genes were purchased from Research Genetics, Inc. (Huntsville, AL). cDNA probes were generated from 1 μg total RNA isolated from five tibial metaphyses from each group of animals by RT (Superscript II, Life Technologies, Inc., Gaithersburg, MD). First strand cDNA probes were primed by addition of oligo(deoxythymidine) and labeled with [α-33P]deoxy-CTP (ICN Bio-medicals, Inc., Costa Mesa, CA). The probes were subsequently purified by passage through a Sephadex G-50 DNA grade column (Amersham Pharmacia Biotech, Uppsala, Sweden). Hybridization was carried out as recommended by the manufacturer’s protocol. After hybridization, the array was washed and wrapped with plastic wrap before placing it in a phosphorimaging cassette containing Cyclone Storage Phosphor screen (Packard, Downers Groves, IL) for 24 h. The array was scanned, a phosphorimager analysis, and results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal structural protein L32.

RNase protection assay

Steady state mRNA levels for PDGF-A were determined using an RNase protection assay according to the manufacturer’s protocol (BD PharMingen, San Diego, CA). Quantitation of protected RNA fragments was performed by phosphorimager analysis, and results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal structural protein L32.

Immunohistochemistry of PDGF-A

Immunohistochemical staining for PDGF-A in paraffin sections (5 μm) of demineralized rat femora was achieved with a rabbit anti-avidin-biotin-peroxidase complex kit (VectorElite, Vector Laboratories, Inc., Burlingame, CA) and a polyclonal antibody specific for PDGF-A (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Incubation with primary antibody (1:33 dilution in Tris-HCl buffer) was performed at 37 C (1 h), followed by incubation at room temperature in goat antirabbit antibody (1 h) and peroxidase-labeled avidin-biotin complex (30 min; VectorElite ABC). Positive PDGF-A staining was localized with the chromagen diaminobenzidine upon incubation with hydrogen peroxide substrate. The specificity of the antibody was confirmed with the omission and increasing dilutions of primary antibody, with staining in tissue from vehicle-treated rats, and with staining performed with a non-PDGF-A rabbit antiserum.

Serum chemistry and PTH

Total serum calcium, phosphate, and magnesium were measured by Central Clinical Laboratory Research at the Mayo Clinic using an automated procedure. Serum PTH was measured using an immunoradiometric assay for rat PTH (Immunotopics International, San Clemente, CA), which has approximately 100% cross-reactivity to hPTH.

Bone histomorphometry

The proximal metaphyses were dehydrated in a graded series of ethanol, infiltrated, and embedded in methylmethacrylate (Fisher Scientific, Fair Lawn, NJ). Tissue sections were cut at 5-μm thickness (Reichert-Jung Model 2065 Microtome, Heidelberg, Germany) and mounted unstained for dynamic cancellous bone measurements. Consecutive sections were stained with toluidine blue for bone cell and

FIG. 1. Continuous, but not pulsatile, PTH increases mRNA levels for PDGF-A.

Rats were treated with vehicle, daily sc injection of hPTH-(1–34) (pulsatile PTH), or ac implanted hPTH-(1–34) osmotic pump (continuous PTH) for 7 d. Tibial metaphyses were removed for total RNA extraction. A, Representative RNase protection assay for PDGF-A and housekeeping genes, L32 and GAPDH. B, Results are expressed in arbitrary densitometric units normalized for the expression of L32 in each group. Continuous PTH significantly increased PDGF-A either expressed per L32 or GAPDH (data not shown). Each bar represents the mean ± SEM (n = 5), a, P < 0.001 compared with vehicle; b, P < 0.01 compared with pulsatile PTH.
peritrabecular fibrosis measurements and with Goldner’s method for photomicrographs. A standard sampling site of 2.8 mm² was established in the secondary spongiosa of the metaphysis 1.5 mm distal to the growth plate. All histomorphometric measurements were carried out with an Osteomeasure image analysis system (OsteoMetrics, Atlanta, GA), and parameters were calculated according to the standardized nomenclature (16). The following parameters were obtained as previously described (11): bone volume per tissue volume, tetracycline and calcein labels, mineral apposition rate, osteoblast surface, osteoclast surface, and fibrotic perimeter. The bone formation rate was calculated as the product of mineral apposition rate and the calcein-labeled perimeter, expressed per bone surface, bone volume, or tissue volume.

**Statistical analysis**

Multiple group comparisons were determined using one-way ANOVA with statistical significance at $P < 0.05$. Differences between pairs of groups were compared by Fisher’s protected least significant difference *post hoc* test. Two-way ANOVA was performed to determine

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**Fig. 2. PDGF-A immunohistochemistry.** Specific staining for PDGF-A was localized to mast cells (arrows) in the femoral metaphysis of rats treated with vehicle (A) and continuous PTH (B). Staining was completely eliminated in sections incubated in the absence of primary antibody or with a non-PDGF-A rabbit antiserum. B, Cancellous bone. Immunohistochemically stained cells were identified as mast cells by metachromatic staining of histamine-containing granules (inset) by 0.1% toluidine blue in the same PTH sections (C).
Continuous PTH induces parathyroid bone disease in rats

We confirmed the differential effect of two types of PTH administration on bone histology (11). As expected, continuous PTH for 7 d induced tibial metaphysis histopathology resembling high turnover parathyroid bone disease. The PTH-treated rats had increased bone formation and resorption and extensive peritrabecular bone marrow fibrosis. The histological changes contrast with the effects of pulsatile administration of PTH, which dramatically increased bone formation without inducing development of peritrabecular fibrosis or abnormal bone resorption.

Increased PDGF-A signaling is associated with parathyroid bone disease

To understand the etiopathogenesis of parathyroid bone disease, we identified differentially expressed genes using cDNA microarrays. Total cellular RNA was isolated from the same region of contralateral tibiae of rats on which histological measurements were performed. The RNA was hybridized with rat gene filter microarrays containing 5531 genes, and data from rats treated with continuous PTH were compared with pulsatile PTH. Approximately 14% of the total genes measured were differentially expressed. We detected increased expression of several growth factors. We focused our attention on one of these, PDGF-A, a known mitogenic and chemotactic factor for fibroblasts. We next verified the differential regulation of PDGF-A expression using an RNase protection assay (Fig. 1A). Pulsatile PTH had no effect on steady-state mRNA levels for PDGF-A, whereas continuous PTH resulted in a statistically significant 3.3-fold increase in the expression of PDGF-A mRNA (Fig. 1B). Additionally, we localized PDGF-A protein in bone by immunohistochemistry. Mast cells in continuously PTH-treated rats stained intensely positive for PDGF-A (Fig. 2). Much weaker staining was observed in growth plate hypertrophic chondrocytes. PDGF staining was weaker yet in osteoblasts, osteoclasts, and fibroblasts.

Trapidil antagonizes PDGF signaling and decreases parathyroid bone disease in rats

Triazolopyrimidine (trapidil) is a competitive inhibitor of PDGF receptor (17) and decreases mRNA levels for PDGF receptor and peptide (18). We examined the effects of PTH and trapidil on BW and serum chemistry (Table 1). Continuous PTH decreased BW compared with the effect of trapidil. Trapidil itself did not have any effect on BW, but it did slightly increase BW in the animals cotreated with PTH. Continuous PTH had no effect on serum concentrations of phosphorus and magnesium and increased serum PTH and calcium levels. Trapidil alone had no effect on serum chemistry, but in combination with PTH it reduced serum calcium.

Table 1. BW, serum chemistry, and bone histomorphometric measurements

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vehicle (n = 9)</th>
<th>Trapidil (n = 10)</th>
<th>PTH (n = 8)</th>
<th>PTH + trapidil (n = 10)</th>
<th>Two-way ANOVA (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial (g)</td>
<td>232 ± 5</td>
<td>233 ± 4</td>
<td>230 ± 4</td>
<td>233 ± 6</td>
<td>NS NS NS</td>
</tr>
<tr>
<td>Final (g)</td>
<td>245 ± 5</td>
<td>252 ± 5</td>
<td>231 ± 4*</td>
<td>249 ± 5b</td>
<td>NS &lt;0.05 NS</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium (mg/dl)</td>
<td>10.26 ± 0.07</td>
<td>10.10 ± 0.05</td>
<td>11.65 ± 0.28*</td>
<td>10.94 ± 0.32a,b,c</td>
<td>&lt;0.001 &lt;0.05 NS</td>
</tr>
<tr>
<td>Phosphorus (mg/dl)</td>
<td>7.89 ± 0.27</td>
<td>7.33 ± 0.29</td>
<td>7.95 ± 0.48</td>
<td>7.46 ± 0.29</td>
<td>NS NS NS</td>
</tr>
<tr>
<td>Magnesium (mg/dl)</td>
<td>2.34 ± 0.05</td>
<td>2.40 ± 0.05</td>
<td>2.49 ± 0.04</td>
<td>2.46 ± 0.07</td>
<td>NS NS NS</td>
</tr>
<tr>
<td>PTH (pg/ml)</td>
<td>19.45 ± 8.37</td>
<td>28.80 ± 9.62</td>
<td>85.29 ± 13.82</td>
<td>108.23 ± 41.06*</td>
<td>&lt;0.001 NS NS</td>
</tr>
<tr>
<td>Bone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>24.88 ± 0.94</td>
<td>24.83 ± 0.87</td>
<td>27.25 ± 1.14</td>
<td>23.79 ± 0.86</td>
<td>NS NS NS</td>
</tr>
<tr>
<td>Tetracycline label (%)</td>
<td>7.86 ± 0.94</td>
<td>9.65 ± 1.76</td>
<td>5.65 ± 2.48</td>
<td>6.91 ± 1.71</td>
<td>NS NS NS</td>
</tr>
<tr>
<td>Calcein label (%)</td>
<td>18.63 ± 2.22</td>
<td>18.32 ± 1.39</td>
<td>48.08 ± 5.35*c</td>
<td>43.24 ± 4.42a,c</td>
<td>&lt;0.001 NS NS</td>
</tr>
<tr>
<td>MAR (µm/day)</td>
<td>1.09 ± 0.05</td>
<td>1.06 ± 0.04</td>
<td>1.29 ± 0.08</td>
<td>1.18 ± 0.07</td>
<td>&lt;0.05 NS NS</td>
</tr>
<tr>
<td>BFR/BS (µm/µm²)</td>
<td>0.29 ± 0.03</td>
<td>0.30 ± 0.03</td>
<td>0.71 ± 0.10*c</td>
<td>0.59 ± 0.06a,c</td>
<td>&lt;0.001 NS NS</td>
</tr>
<tr>
<td>BFR/BV (%)</td>
<td>1.04 ± 0.11</td>
<td>0.99 ± 0.08</td>
<td>2.30 ± 0.31*c</td>
<td>2.06 ± 0.18a,c</td>
<td>&lt;0.001 NS NS</td>
</tr>
<tr>
<td>BFR/BV (%)</td>
<td>0.26 ± 0.03</td>
<td>0.25 ± 0.02</td>
<td>0.63 ± 0.09</td>
<td>0.49 ± 0.05a,c</td>
<td>&lt;0.001 NS NS</td>
</tr>
</tbody>
</table>

Data represent the mean ± SEM. BV/TV, bone volume per tissue volume; MAR, mineral apposition rate; BFR, bone formation rate; BS, bone surface; BV, bone volume; TV, tissue volume.  

P < 0.05 vs. trapidil. 

P < 0.05 vs. PTH. 

P < 0.05 vs. vehicle.
bone resorption (Figs. 4B and 5C) and induced extensive peritrabecular fibrosis (Figs. 4C and 5C). Trapidil decreased osteoclast perimeter and peritrabecular fibrosis induced by PTH by 73% and 63%, respectively. Two-way ANOVA revealed an interaction between PTH and trapidil on osteoclast and peritrabecular fibrotic perimeters, confirming that trapidil antagonized the detrimental skeletal effects of continuous PTH, but had no effect on normal rats. Combination treatment with PTH and trapidil resulted in histological changes (Table 1 and Fig. 5D) similar to those observed after pulsatile PTH treatment, as shown by increases in bone formation and osteoblast surface. These data suggest that trapidil, by antagonizing PDGF-A, selectively reduces skeletal pathology induced by continuous infusion of PTH.

Discussion

In this study we identify PDGF-A as a potential causative factor for parathyroid bone disease in an animal model for HPT. PDGF-A expression increases in skeletal tissue in this model. Treating PTH-infused animals with trapidil, an inhibitor of PDGF signaling, provides a highly effective intervention for preventing the skeletal disorders, leading to a striking reduction of peritrabecular fibrosis and osteoclastic bone resorption.

PTH has incongruous effects on bone metabolism depending on the pattern of exposure to the hormone (19–21). Pulsatile PTH increases bone formation, whereas continuous PTH stimulates bone resorption as well as bone formation and induces marrow fibrosis. The signal transduction pathways of PTH begin with the hormone binding to the highly conserved PTH/PTH-related peptide receptor at the osteoblast surface. Upon binding to its receptor, early signaling events are sufficient to induce the anabolic response. However, late events associated with continuously elevated PTH lead to parathyroid bone disease. Therefore, understanding the differential gene expression of these two regimens is essential for determining what signals mediate PTH-induced parathyroid bone disease. Identification of genes induced by continuous vs. pulsatile PTH was performed using cDNA microarray, a powerful tool for profiling gene expression. Using this approach, we obtained preliminary evidence that PDGF-A mRNA becomes elevated by continuous, but not pulsatile PTH. These gene array data were confirmed using an RNase protection assay.

PDGF is a homo- or heterodimer polypeptide encoded by two distinct genes, PDGF-A and PDGF-B (22). The PDGF-A and PDGF-B chains share 56% homology and join by disulfide bond to form three different dimers, AA, AB, or BB (23, 24). Cellular responses are mediated via two high affinity receptor subunits, α and β. PDGF-A binds primarily to the α-receptor, whereas PDGF-B binds to the α- or β-receptor (25). PDGF has potent mitogenic and chemotactic actions on mesenchymal cells. PDGF plays a role in physiological repair mechanisms and pathogenesis of proliferative diseases, including tumorigenesis, atherosclerosis, inflammatory disorders, and, most relevant to the present investigation, fibrosis (26, 27).
[3H]Thymidine radioautography was used to determine the origin of the peritrabecular fibroblasts induced by continuous infusion of PTH and indicated extensive proliferation of the fibroblasts lining bone surfaces (28). This finding suggests a role for a PTH-induced growth factor, possibly PDGF-A, in the recruitment to bone surfaces and expansion of fibroblast populations by PTH.

PDGF-A peptide was localized to mast cells by immunohistochemistry. This intriguing result suggests that cytokines that are released by mast cells, including PDGF, mediate parathyroid bone disease. This mechanism of action is supported by evidence implicating mast cells in pathological fibrosis as well as pathological bone resorption (29–31). Additionally, HPT patients have increased numbers of mast cells in the vicinity of trabecular surfaces of cancellous bone (32), and PTH is reported to induce mast cell degranulation (33).

Trapidil, originally developed as a vasodilator and antiplatelet agent, has proven to be clinically effective in the treatment of coronary heart disease (34). Trapidil has been reported to antagonize PDGF signaling by acting as a PDGF receptor antagonist and by reducing PDGF and PDGF receptor gene expression (17, 18). Trapidil has been reported to have additional actions in vitro; including inhibition of phosphodiesterase (35), thromboxane A2 (36), and CD40 signaling (37), and direct activation of protein kinase A signaling (38).

The observed decreases in mRNA levels for PDGF-A and PDGF receptor-α demonstrate that trapidil antagonizes PDGF-A signaling in PTH-treated rats. The lack of an effect of trapidil on normal rats indicates that the drug is unlikely to have inhibited phosphodiesterase or have activated protein kinase A signaling, because these actions would have been expected to have dramatic effects on bone turnover (39, 40).

PTH treatment decreased mRNA levels for thromboxane A2 (data not shown), so it is unlikely that a further decrease by trapidil could be responsible for the actions of the drug on bone. Finally, we did not detect an effect of trapidil on mRNA levels for TNF-α, IL-6, and IL-12 (data not shown), genes reported to be down-regulated in response to blockade of the CD40 pathway. Although we cannot rule out the possibility of multiple pathways of action, we have established that trapidil antagonizes PDGF signaling and have uncovered no evidence to implicate alternative pathways.

Treatment of animals with trapidil did not alter serum PTH levels, demonstrating that the drug did not act by ac-
celerating the metabolism of the hormone. Trapidil, however, did decrease serum calcium in PTH-treated rats. This reduction of serum calcium was probably due to an inhibitory effect of trapidil on bone resorption, as shown by a decrease in osteoclast surface. There are a variety of mechanisms by which PDGF-A signaling could act as an indirect factor in PTH-induced bone resorption. For example, fibroblasts have been reported to express Rankl, a critical factor in the differentiation of osteoclasts (41). The combination of marrow fibrosis induced by PDGF-A and additional factors that induce Rankl expression could be responsible for the focal bone resorption associated with hyperparathyroidism. This possibility is consistent with clinical observations; peritrabecular fibrosis and increased bone resorption generally occur in combination in patients with renal osteodystrophy (10). Alternatively, PDGF-A signaling could play a role in the release from mast cells of one or more cytokines that stimulate osteoclast function (42). Further research will be necessary to test these and other possibilities.

All three isoforms of PDGF were shown to induce the proliferation of osteoblastic cells in culture (PDGF-BB→PDGF-AB→PDGF-AA) (43, 44). In cultured calvariae, PDGF increases the number of cells capable of synthesizing collagen and inhibits matrix synthesis in intact calvariae (45). Other studies have reported that the effects of PDGF on in vitro mineralization depend upon the duration of exposure to the cytokine; pulsed exposure increases mineralization, whereas continuous exposure antagonizes mineralization (31).

Continuous infusion of PTH resulted in tibial metaphysis histological changes similar to those described in a previous report, including multilayers of fibroblasts lining trabeculae (11). Trapidil was found to inhibit BALB/c 3T3 fibroblast proliferation induced by PDGF (46), and we demonstrated that trapidil markedly reduced peritubular fibrosis. Trapidil was also reported to strongly decrease gene expression of PDGF-α and -β receptors and to moderately suppress mRNA levels of PDGF-A and -B in injured rat arteries (18). We have found that trapidil similarly decreases mRNA levels for PDGF-A and PDGF-α receptor in bone.

In conclusion, these studies provide evidence that PDGF-A signaling is critical to the pathogenesis of parathyroid bone disease. Trapidil, an antagonist of PDGF signaling, suppressed the development of peritubular fibrosis and osteoclastic bone resorption while allowing the elevated bone formation to continue. The latter finding suggests that increased PDGF-A signaling is unnecessary for the initial stimulation of bone formation by PTH. The beneficial effects of trapidil on bone with no adverse effects at the dosage tested in this study suggest a use for PDGF antagonists in the treatment of parathyroid bone disease. Whether trapidil can reduce established marrow fibrosis requires further investigation.

**Acknowledgments**

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