Down-regulation of human osteoblast PTH/PTHrP receptor mRNA in end-stage renal failure

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Background. Resistance to the action of parathyroid hormone (PTH) has been demonstrated in end-stage renal failure and is considered to be important in the pathogenesis of secondary hyperparathyroidism. The mechanism of resistance is unknown. However, altered regulation of cellular PTH/PTH-related protein (PTH/PTHrP) receptor (PTH1R) has been assumed to be important.

Methods. We have used in situ hybridization to examine PTH1R mRNA expression by osteoblasts in human bone and have compared the expression in high- and low-turnover renal bone disease, high-turnover nonrenal bone disease (healing fracture callus and Pagetic bone), and normal bone. Bone biopsies were formalin fixed, ethylenediaminetetraacetic acid decalcified, and paraffin wax embedded. A 1.8 kb PTH1R cDNA probe, labeled with $^{35}$S, was used, and the hybridization signal was revealed by autoradiography. The density of signal over osteoblasts was quantitated using a semiautomated Leica image analysis software package.

Results. The mean density of PTH1R mRNA signal over osteoblasts in renal high-turnover bone was only 36% of that found in nonrenal high-turnover bone ($P < 0.05$) and 51% of that found in normal bone ($P < 0.05$). Osteoblast PTH1R mRNA signal in adynamic bone from individuals with diabetes mellitus was 28% of normal bone ($P < 0.05$) and 54% of that found in renal high-turnover bone ($P < 0.05$).

Conclusions. These results demonstrate a down-regulation of osteoblast PTH1R mRNA in end-stage renal failure in comparison to normal and high-turnover bone from otherwise healthy individuals, and provide an insight into the mechanisms of “skeletal resistance” to the actions of PTH.

Bone is influenced by the cumulative, long-term effects of metabolic derangement, and thus, the long-standing alterations in mineral metabolism generated by renal failure have profound effects on the skeleton. Abnormalities of bone turnover are characteristic of renal osteodystrophy. High turnover bone disease is typified by high numbers of osteoblasts and osteoclasts with high bone formation rates. Patients typically have elevated levels of serum parathyroid hormone (PTH). In contrast, abnormally low numbers of osteoblasts and osteoclasts are found in low turnover bone lesions (adynamic and osteomalacia) with low bone formation rates and PTH levels within or just above the normal range. PTH levels are usually elevated in end-stage renal disease as a probable consequence of decreased 1,25-dihydroxyvitamin D$_3$, hyperphosphatemia, hypocalcemia, and end-organ resistance to PTH. Resistance to the action of PTH in patients with chronic renal failure has been known for many years [1, 2]. These findings have been confirmed by the demonstration of a diminished calcemic response to PTH stimulation in both uremic dogs [3] and rats [4], while isolated perfused bones of uremic dogs have a blunted cAMP response to PTH [5]. Furthermore, recent histomorphometric studies of human bone biopsies have shown that serum intact PTH (iPTH) levels several times greater than normal are required to maintain parameters of bone turnover within the normal range [6–11]. Such data have led several authors to conclude that there must be altered regulation of PTH receptors, resulting in “down-regulation” or “desensitization” [7, 12]. The etiology of skeletal resistance to PTH in renal failure is unknown. Hyperphosphatemia [12, 13], deficiency of vitamin D$_3$ [14–19], uremic toxins (abstract; Dunlay et al, Kidney Int 35:426, 1989) [4, 19], hypercalcitoninemia [20], and hyperparathyroidism (abstract; ibid) [3, 19, 21] have all been postulated as factors that modulate PTH receptor function in vivo causing skeletal resistance in renal failure.

Parathyroid hormone acts on target tissues via a specific receptor that recognizes not only PTH but also PTH-
related protein (PTHrP), the PTH/PTHrP receptor (PTH1R) [22]. Receptor regulation may occur at several points, namely transcription, translation, receptor expression, ligand affinity, and second-messenger/effector activation [23]. With the cloning of the rat PTH1R [24, 25], attention has focused on transcriptional regulation of the receptor in renal failure in rats. Urena et al. isolated total RNA from bone and kidney of uremic rats and found a decrease in PTHR1 mRNA compared with normal rats [26]. Other authors have also recently reported a down-regulation of PTH1R mRNA in kidney, liver, and heart from uremic rats [27–29]. Although histomorphometric data suggest that human bone cells exhibit PTH resistance in renal failure [6–10], there is no explanation for this at the cellular level in vivo. Cloning of the human PTH1R gene [24, 25] and the development of molecular techniques such as in situ hybridization (ISH) have made it possible to address questions that are fundamental in understanding renal bone cell biology in vivo. Therefore, the aim of this study was to investigate, using ISH, whether there is down-regulation of the PTH1R mRNA in human osteoblasts in renal failure as compared with normal osteoblasts and osteoblasts in other high-turnover bone states.

METHODS

Subjects

This study comprised 13 individuals with renal failure (8 male and 5 female, mean age of 46.1 years, range 27 to 63 years). Of these, three individuals were in end-stage renal failure (ESRF) and had a trans-iliac bone biopsy under general anesthetic at the time of Tenckhoff catheter insertion; the remaining 10 individuals were receiving continuous ambulatory peritoneal dialysis (mean duration of 25.5 months, range 1 to 48 months). The cause of renal failure in two individuals was unknown. Two had autosomal dominant polycystic kidney disease. Two individuals were recorded as having hypertensive renal disease. One had chronic pyelonephritis, and six had diabetic nephropathy. All subjects were taking calcium carbonate with meals as a phosphate binder, and none were taking aluminum-containing phosphate binders. No subject had been treated with vitamin D or had undergone parathyroidectomy. None of the females had received hormone replacement therapy. Blood sampling was carried out on the same day as bone biopsy to allow measurement of ionized calcium, serum phosphate, serum iPTH, and serum 1,25(OH)2D3. The study also comprised individuals with normal renal function with Paget’s disease (N = 2, both male, 61 and 58 years old) and healing bone fracture (N = 2, both male, 42 and 47 years old), which comprised the nonrenal high-turnover group and specimens of normal bone from age-matched individuals obtained from the iliac crest at the time of bone grafting (N = 5).

Sources and preparation of bone tissue

Specimens examined were as follows: 13 iliac bone biopsies from individuals with ESRF, which showed no evidence of a mineralization defect or aluminum deposition; 2 iliac bone biopsies from patients with Paget’s disease; 2 healing fracture callus specimens; and 5 samples of normal iliac bone. Bone trephines were split longitudinally into two portions. One portion was fixed in ethanol and embedded in methylmethacrylate resin. From these resin blocks, 7 μm sections were cut using an LKB macrotome, stained free-floating in toluidine blue, and mounted on glass slides. All renal patients received 1 g of oral tetracycline 14 and 4 days prior to bone biopsy. For these samples, additional 15 μm sections were cut for examination of tetracycline labeling using ultraviolet epi-illumination microscopy. The criteria for classification as either adynamic or high-turnover bone disease have been published previously [30]. The second portion was fixed for 24 hours in 10% (vol/vol) neutral-buffered formalin and then decalcified in 20% wt/vol ethylenediaminetetraacetic acid (EDTA; pH 7.2) until radiologically decalcified (10 to 14 days). Following decalcification, the tissue was processed routinely into paraffin wax and 5 μm sections mounted onto silanated slides [31] for ISH.

In situ hybridization

Probe. A human cDNA probe (1.8 kb) to PTH1R (a generous gift from Dr. Schipani, Boston, MA, USA) was random prime labeled to a specific activity of approximately 1 × 106 cpm/g using 35S dCTP. For control purposes, similarly sized fragments of lambda DNA labeled to the same specific activity were used.

Hybridization. The prehybridization treatments were as detailed previously [32]. Briefly, these included sequential immersion in 0.2N HCl (20 min), 0.2 × standard saline citrate (SSC = 0.15 mol/L NaCl and 0.015 mol/L sodium citrate, 10 min); g/mL of proteinase K in 50 mmol/L Tris-HCl, pH 7.5, one hour at 37°C; 0.2% (wt/vol) glycine in phosphate-buffered saline (PBS); and 0.4% (wt/vol) paraformaldehyde in PBS, pH 7.0 (20 min), and freshly prepared 0.25% (vol/vol) acetic anhydride in 0.1 mol/L triethanolamine, pH 8. Following proteinase treatment, serial sections were reacted with 1 mg/mL of RNAs A in 0.5 × SSC for one hour at 37°C. All sections were prehybridized for one hour at 37°C in 50% formamide, 1 mg/mL of bovine serum albumin, 0.02% (wt/vol) Ficoll, 0.02% (wt/vol) polyvinyl pyrrolidone, 0.6 mol/L NaCl, 0.2 mg/mL of sheared salmon sperm DNA, 10 mmol/L Tris (pH 7.4), 0.5 mmol/L EDTA, 10 mmol/L dithiothreitol (DTT), and 10% (wt/vol) dextran sulfate. Hybridization with heat denatured
**Quantitation.** Analysis of autoradiographic signal over bone cells was performed using a Quantimet™ semiautomated image analysis system incorporating software designed in conjunction with Leica Software Systems (Milton Keynes, UK). Analysis was performed by two investigators (P.M. and J.A.H.). Using t-test and analysis of variance (ANOVA), there was no significant difference between the two observers \( (P < 0.05) \). ISH was performed four times on a series of biopsies, and no significant difference was found between ISH runs \( (P < 0.05) \). Signal density (grains per unit area of cell) was analyzed for osteoblasts. For each biopsy, approximately 10 random high-power fields were examined or as many as possible if less than 10. All osteoblasts within each high-power field were identified and their area and that portion of that area covered by signal measured in arbitrary pixel units. This represents “Signal Density.” Background signal over matrix and noncellular areas of bone was measured in two random high-power fields in the same manner. Results were recorded as the mean density of signal over cells in all fields, minus the background signal.

**Biochemistry.** Serum iPTH levels were measured in all renal patients using a commercially available two-site immunoradiometric assay (IRMA) for the measurement of PTH (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA). Serum levels of 1,25(OH)\(_2\)D\(_3\) were measured using a method previously described [33]. Serum phosphate was measured by colorimetric assay using a Hitachi 747 Autoanalyzer (Nissei Sangyo Co., Ltd., London). Ionized calcium was determined using a Radiometer ICA2 electrode.

**Statistical analysis**

Summary statistics in the form of means and 95% confidence intervals (95% CI) were calculated for mRNA signal density for normal bone, Paget’s disease and healing fracture, and renal high- and low-turnover groups, and data are represented as the mean and 95% CI. Comparisons between histologic groups were carried out using the Mann–Whitney U-test. Differences were considered significant at a value of \( P < 0.05 \). In the renal high- and low-turnover groups, Spearman’s rank-order correlation was used to investigate the relationship between serum iPTH, serum phosphate, ionized calcium, serum 1,25(OH)\(_2\)D\(_3\), duration of dialysis, and the age of subject at the time of bone biopsy and PTH1R mRNA signal.

**RESULTS**

**Biochemistry**

The mean serum iPTH for the group with hyperparathyroid bone disease \( (N = 9) \) was 644 pg/mL (range 180 to 1700 pg/mL) and 85 pg/mL (range 30 to 148 pg/mL) for those with diabetes and adynamic bone \( (N = 4) \). The normal range for serum iPTH was 10 to 65 pg/mL. The mean serum 1,25(OH)\(_2\)D\(_3\) for the hyperparathyroid bone group was 7.8 pg/mL (range 0 to 18 pg/mL) and 5.8 pg/mL (range 3 to 10 pg/mL) for the group with adynamic bone and diabetes mellitus. Levels of 1,25(OH)\(_2\)D\(_3\) greater than 20 pg/mL are considered normal. The mean serum phosphate for the hyperparathyroid bone group was 2.1 mmol/L (range 1.2 to 3.07 mmol/L), and that for the diabetic-adynamic bone group was 1.7 mmol/L (range 1.54 to 2.16). The normal range was 0.75 to 1.5 mmol/L. Mean ionized calcium for the hyperparathyroid bone group was 1.24 mmol/L (range 1.01 to 1.41 mmol/L) and for the diabetic-adynamic bone group was 1.2 mmol/L (range 1.12 to 1.22 mmol/L). The normal range is taken as 1.12 to 1.33 mmol/L. The complete biochemical data are shown in Table 1.

**Cellular localization of PTH1R mRNA**

PTH1R mRNA hybridization signal was generally greatest over osteoblasts with variation in signal intensity seen in the different bone states studied (Fig. 1). In addition, in individual biopsies, some variability in intensity of signal was apparent with “plump” osteoblasts in areas of active matrix synthesis most positive. There was no difference between cortical and trabecular osteoblasts. No signal was seen over flat resting surface cells, and osteocytes were not convincingly positive except in healing fracture callus. This pattern of signal expression was seen in all bone studied, although the level of signal differed. Flat elongated fibroblast-like cells in areas of peritrabecular fibrosis (in the renal bone biopsies) were positive, although much less than osteoblasts. This was particularly the case when peritrabecular fibrosis was adjacent to trabecular surfaces, which were being actively remodeled. Scattered unidentifiable bone marrow cells were positive. Clearly, the localized hybridization signal was also seen over actively resorbing osteoclasts.
in renal hyperparathyroid bone, healing fracture callus, and particularly in Pagetic bone (Fig. 2). In Pagetic bone, the hybridization signal was often greater over osteoclasts than over osteoblasts. No identifiable osteoclasts were seen in normal bone.

Quantitation of PTH1R mRNA expression

PTH1R mRNA expression in osteoblasts was significantly less in renal high-turnover bone (mean 3.70, 3.40 to 4.13, 95% CI) than either the fracture and Pagetic bone (mean 10.30, 9.72 to 10.87, 95% CI) or normal bone (mean 7.24, 6.41 to 8.08, 95% CI, \( P < 0.05 \)). In diabetic-adynamic bone, PTH1R mRNA expression (mean 1.99, 1.27 to 2.70, 95% CI) was significantly less than in high-turnover renal bone and normal bone (\( P < 0.05; \) Fig. 3). There were no significant correlations between PTH1R mRNA signal and serum PTH (\( r = 0.58 \)), serum 1,25(OH)\(_2\)D\(_3\) (\( r = 0.24 \)), serum phosphate (\( r = 0.34 \)), ionized calcium (\( r = 0.50 \)), age at time of biopsy (\( r = -0.38 \)), or dialysis duration (\( r = -0.26 \)) when analyzed using Spearman’s rank-order correlation in the 13 ESRF patients examined (Fig. 4).

DISCUSSION

Parathyroid hormone has a central role in renal osteodystrophy, and the cloning of the PTH1R gene has made it possible to investigate the possibility of altered receptor mRNA regulation. Although several authors have suggested that abnormal regulation of the PTH1R by bone cells in patients with ESRF is likely to be important in the pathogenesis of skeletal resistance to PTH found in these patients [7, 11], to our knowledge, this is the first time that a reduction in steady-state levels of PTH1R mRNA has been demonstrated in human osteoblasts. PTH1R mRNA in vivo has been shown to be down-regulated in uremic rat kidney [26, 27], liver [28], and heart [29]. Interestingly, Urena et al have also examined bone from two uremic rats and found a PTH1R mRNA/28S ratio of 0.78 in normal animals compared with 0.59 in rats with chronic renal failure illustrating a down-regulation of receptor mRNA in skeletal tissue [26]. In this study of osteoblasts in human bone, the mean PTH1R mRNA signal density in high-turnover renal osteodystrophy samples was 3.70 (3.40 to 4.13, 95% CI) and in diabetic-adynamic renal bone was 1.99 (1.27 to 2.70, 95% CI). By contrast, in the nonrenal high-turnover specimens, the mean signal density was 10.30 (9.72 to 10.87, 95% CI) and in normal bone was 7.24 (6.41 to 8.08, 95% CI). Hence, renal high-turnover osteoblasts in vivo had levels of PTH1R mRNA expression that were 36 and 51% of levels found in nonrenal high-turnover and normal bone, respectively. Osteoblasts from diabetic-adynamic bone had receptor mRNA expression 28% of normal osteoblasts and 54% of the expression found in high-turnover renal bone. Our data show down-regulation of PTH1R mRNA in the PTH target tissue, bone. The decrease in PTH1R expression at the level of the osteoblast may represent the molecular basis for the resistance of the skeleton to PTH in uremic patients. It must be noted that we have shown down-regulation of receptor mRNA and not of the receptor protein itself, although it is probable that the expression of the receptor protein is also down-regulated in uremia. However, this still remains to be demonstrated.

In a study of this nature, where the number of biopsies is small, it is difficult to draw definite conclusions about the roles of serum PTH, ionized calcium, serum phosphate, serum vitamin D\(_3\), and uremic factors in the pathogenesis of PTH1R modulation, in particular the down-regulation of receptor mRNA. There is general agreement in the literature regarding the negative regulatory effect of PTH on PTH1R expression and cAMP production in vitro [34–41]. More recently, the expression of PTH1R receptor mRNA in vitro has been examined and found to be reduced in response to PTH stimu-
Fig. 1. Photomicrograph showing PTH1R mRNA expression [visualized as black (silver) autoradiographic grains] by osteoblasts (arrow) lining the bone surface in (a and b) renal hyperparathyroid bone (a, magnification ×50; b, magnification ×200), (c and d) normal bone (magnification ×250), and (e and f) normal healing fracture callus (e, magnification ×50; f, magnification ×200). Note the increase in signal seen in nonuremic osteoblasts compared with those in renal bone.

lation [42–46]. The data obtained from experimental animals, however, have not been as clear cut. A reduction of increased PTH levels to normal did not improve the blunted calcemic response to PTH in azotemic animals (abstract; Bover et al, XIth International Congress of Nephrology, Jerusalem, Israel, June 13–18, 1993, p 453). In addition, Urena et al have shown that thyroparathyroidectomy does not prevent renal PTH1R mRNA down-regulation in uremic rats, suggesting that hyperparathyroidism is not essential for down-regulation to occur [47]. Others have found that selective parathyroidectomy did normalize receptor mRNA levels in uremic rat kidney, liver, and heart [27–29]. This same group found that parathyroidectomy in animals without renal failure did not increase the expression of PTH1R mRNA [48]. In our present study, the majority of individuals had serum PTH levels that were elevated above normal, although those individuals with the higher levels were those with high-turnover bone disease and had relatively higher levels of PTH1R mRNA expression. This would argue against PTH being the cause of receptor mRNA down-regulation.

The role of 1,25(OH)2D3 in vivo is to improve or abolish resistance to PTH in uremic individuals [14–18]. Studies in vitro reveal that the cAMP response of bone cells from vitamin D-deplete animals to PTH stimulation is
Fig. 2. Photomicrograph showing PTH1R mRNA expression by osteoclasts in renal hyperparathyroid bone (a, magnification ×100) and Pagetic bone (b, magnification ×100). Note the increase in hybridization signal in Pagetic osteoclasts.

Fig. 3. Levels of osteoblast PTH1R mRNA signal in different bone states. PTH1R mRNA hybridization signal in osteoblasts from fracture callus (F1-2), Pagetic bone (P1-2), normal bone (N1-4), renal hyperparathyroid (H1-9), and diabetic-adynamic bone (A1-4). Mean signal density values (per unit area of cell) with 95% CI are presented for each individual.
increased after treatment of those bone cells with vitamin D [49]. However, the majority of in vitro experiments reveal a reduction in cAMP response [50] and a reduction in receptor number and receptor mRNA [51] after treatment with 1,25(OH)$_2$D$_3$. It would seem that the level of vitamin D depletion and the presence of uremia are important factors in determining the effect of vitamin D. No significant correlation was found between serum 1,25(OH)$_2$D$_3$ and receptor mRNA signal in the group of individuals examined in this study.

In vivo studies carried out in animals with renal failure have implicated hyperphosphatemia as an important factor in the development of PTH resistance [13, 52]. In addition, in vitro experiments have shown that both high [12, 53, 54] and low [55] extracellular levels of phosphate reduce either cAMP or calcium efflux from bone in response to PTH stimulation. No significant correlation was found between serum phosphate and receptor mRNA signal in the group of individuals examined in our study.

Intracellular calcium levels have been felt to be important in the down-regulation of PTH1R mRNA in rat kidney, liver, and heart [28, 29, 48, 56]. However, in this present study, we found no significant correlation between extracellular ionized calcium and receptor mRNA signal.

In the present study, the duration of peritoneal dialysis was significantly different between the high- and low-turnover bone groups, with the adynamic group being on dialysis for longer ($P < 0.05$). Other studies have indicated that continuous ambulatory peritoneal dialysis is associated with adynamic bone disease [57], and in our study, adynamic bone is associated with lower levels of receptor mRNA. It must be noted that all of those individuals with adynamic bone had diabetes mellitus, which is itself associated with adynamic bone disease, and diabetes mellitus has also been shown to be associated with the down-regulation of the PTH1R mRNA in renal tissue [56]. Thus, it remains unclear whether duration of dialysis, the presence of diabetes mellitus,
or the low bone-turnover state per se is crucial in this situation.

Local factors in the bone environment are almost certainly involved in the modulation of osteoblast PTH1R expression. In vitro, tumor necrosis factor-α [58–61], interleukin-1 [58, 62], transforming growth factor-β [63–66], epidermal growth factor [67], and PTHrP [68] have all been shown to affect the expression or function of the PTH1R in osteoblast-like cells. In addition, type I collagen and matrix proteins are known to influence receptor expression [69, 70]. The importance of these local factors is illustrated in the cases of Paget’s disease and fracture healing, situations in which systemic factors are generally unaltered through bone remodeling, and from this study PTH1R mRNA expression is greatly increased. Therefore, alterations in the expression of these locally acting growth factors and cytokines in uremia may be important in explaining the altered expression of PTH1R mRNA in renal failure.

In contrast to many previous studies, but consistent with data by Tong et al (abstract: J Bone Miner Res 10:5278, 1995), who showed that isolated mouse osteoclasts expressed PTH1R mRNA, human osteoclasts (in high-turnover bone states) were positive for PTH1R mRNA. This supports previous in vitro evidence that osteoclasts have the potential to respond to PTH directly [71–73]. In fracture callus and renal high-turnover bone expression of receptor mRNA was less than or equal to that of osteoblasts. However, in Pagetic osteoclasts the expression of receptor mRNA was higher than that by osteoblasts and is consistent with the altered gene expression previously reported [74–76] and may be important in maintaining the Pagetic phenotype.

In conclusion, our data show that there is a reduction in steady-state levels of the PTH1R mRNA in human osteoblasts in vivo in ESRF, and this may represent the Int


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