Kidney International, Vol. 56, Suppl. 73 (1999), pp. S-8–S-13

Cellular mechanisms of renal osteodystrophy

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Cellular mechanisms of renal osteodystrophy. Renal osteodystrophy affects all patients with end-stage renal failure, resulting in significant skeletal and extra-skeletal morbidity. The patterns of disease seen in bone are the result of changes in calcium, phosphate, parathyroid hormone (PTH), and vitamin D metabolism, as well as the effects of uremia. Standard histological techniques, however, give little insight into the altered biological activity or mechanisms of disease at the cellular level. In order to examine the cellular abnormalities in renal bone disease we have performed a series of *in situ* hybridization studies to examine renal bone cell expression of genes for PTH receptor (PTHR1), transforming growth factor β (TGF-β) and insulin growth factor 1 (IGF-I). PTHR1 mRNA was expressed predominantly by osteoblasts, but also by resorbing osteoclasts, suggesting that these cells may be stimulated directly by PTH. Semi-quantitative analysis of gene expression showed downregulation of PTHR1 mRNA by osteoblasts in renal bone compared with normal, fracture and Pagetic bone. This may be important in the pathogenesis of skeletal resistance seen in end-stage renal failure, altering the "threshold" at which PTH has its effects on bone cells. TGF-β and IGF-I mRNA expression was also decreased, suggesting that synthesis of these factors, postulated to be mediators of PTH, is also downregulated.

Renal osteodystrophy is the term used to describe the complex metabolic bone disorders that occur as a complication of renal failure. Abnormalities of bone and mineral metabolism are responsible for a significant proportion of morbidity experienced by individuals with renal failure and occur early in the course of renal failure. As many as 50% of individuals have abnormal bone histology when the glomerular filtration rate (GFR) is reduced by 50% [1] and almost all individuals have it at the start of dialysis [2]. Bone pain and tenderness are the most common symptoms and may be severe and incapacitating. Renal osteodystrophy is the result of prolonged and severe metabolic derangement. In bone the mode of response to this derangement is limited and manifested by an alteration in the number of remodeling sites and in the effectiveness and duration of each phase of the remodeling cycle [3]. Renal osteodystrophy is diagnosed on the basis of bone biopsy examination. Since the changes occurring in bone cells of patients with renal failure are the cumulative result of complex interactions between numerous systemic factors and local factors, the bone biopsy remains the only unequivocal means of evaluating both the type and severity of disease [4, 5]. Standard histologic techniques, however, give little insight into the altered biological activity or mechanisms of disease at the cellular level which are necessary for understanding the nature of disease processes and for developing effective treatments. The recent development of *in situ* molecular techniques (for example, *in situ* hybridization and *in situ* reverse transcriptase PCR) has provided an opportunity for greater understanding of the mechanisms regulating bone cell function and the effect of alteration of these processes in disease.

Key words: end-stage renal disease, IGF-I, osteoblasts, parathyroid hormone receptor, TGF-β.

BONE REMODELING AND RENAL OSTEODYSTROPHY

Renal osteodystrophy is a disorder of bone remodeling. Bone remodeling is a complex dynamic process controlled both temporally and spatially to achieve a balance between the coupled processes of osteoblastic bone formation and osteoclastic bone resorption. It is a multistep process involving osteoclast activation, bone resorption, osteoblast activation and finally bone formation. Each step is regulated by the interplay of systemic hormones (parathyroid hormone [PTH], vitamin D, steroid hormones) and locally produced cytokines and growth factors [6]. The net response of bone cells represents the summation of these inputs. However, the exact mechanism by which the coupled processes of bone resorption and formation are initiated, propagated and terminated at specific sites is not fully understood, partly because of the difficulty in studying this process *in vivo*. Disruption at the various stages of the remodeling cycle results in alteration of bone state. Uncoupling of bone formation can result in either bone loss, as when resorption outpaces formation (e.g., osteoporosis), or bone gain, as when formation outpaces bone resorption (e.g., osteopetrosis).

Renal osteodystrophy occurs as a consequence of a disruption of the remodeling cycle. However, it is not

a uniform bone disorder. Based on histomorphometric findings renal bone disease is classified into two main groups: high and low turnover bone disease [2, 7]. High turnover disease includes moderate and severe hyperparathyroidism characterized by increases in the numbers of osteoblasts and osteoclasts with high rates of bone formation. Typically patients have high levels of serum PTH [4, 8]. Low turnover disease includes adynamic bone characterized by decreased numbers of osteoblasts and osteoclasts together with decreased (frequently zero) rates of bone formation. Patients with adynamic bone have relatively low levels of serum PTH which may be within or just above the normal physiological range [4, 9].

Understanding the mechanisms by which these changes occur necessitates examining the role of calcitropic hormones, how they exert their effects at cellular levels, the resulting modifications in cell function, and the role of cytokines and growth factors in the renal bone microenvironment. Routine histology and histomorphometry give little information about these processes. In an attempt to address some of these questions we have applied *in situ* hybridization to the study of renal osteodystrophy.

SKELETAL RESISTANCE TO PTH

Many of the factors implicated in the pathogenesis of renal osteodystrophy, including hyperphosphatemia, hyperparathyroidism, hypocalcemia, and uremic factors, may affect bone cells directly or indirectly and hence may contribute to disruption of the remodeling cycle. Perhaps one of the major factors in this context, that plays both a key role in bone turnover and renal osteodystrophy, is PTH. PTH is the major stimulant of altered remodeling in uremic patients. At the same time, however, a well-defined but poorly understood PTH resistance occurs in uremia.

Skeletal resistance to the calcemic actions of PTH in renal failure was initially described by Evanson in 1966 [10] and was later felt to be important in the development of secondary hyperparathyroidism by Llach [11], who found a delayed calcemic recovery from ethylenediaminetetraacetic acid (EDTA)-induced hypocalcemia in patients with mild renal failure, despite the fact that these patients had higher than normal levels of PTH. Massry et al [12] used a parathyroid extract infusion to study the calcemic response to PTH in 105 individuals with varying degrees of renal failure. The response was reduced in all renal subjects compared to normals. These findings have been confirmed by the demonstration of a diminished calcemic response to PTH stimulation in both uremic dogs [13–16] and rats [17–22], while isolated perfused bone from uremic dogs showed a blunted cAMP response [23]. Resistance to the effect of PTH has also been identified in uremic rat growth plate cartilage [24]. Such studies suggest that in renal failure there is a degree of resistance to the actions of PTH. This has been confirmed by recent reports showing that elevated serum PTH of between 2 and 4 times that of normal are required to maintain bone cell numbers and parameters of bone turnover within the normal range [7, 8, 25–29].

The pathogenesis of skeletal resistance remains unclear. It has been hypothesized that altered regulation of PTH receptors may occur resulting in downregulation or desensitization [7, 30]. Receptor regulation may occur at several points, namely transcription, translation, receptor expression, ligand affinity and second messenger/effector activation. With the cloning of the rat PTH1R gene [31] attention has focused on the transcriptional regulation of the receptor in renal failure in rats. Urena and colleagues isolated total RNA from bone and kidney of uremic rats and found a decrease in PTH/PTHrP receptor mRNA compared to normal rats [32, 33]. Other workers have also reported a downregulation of PTH/PTHrP receptor mRNA in kidney, liver and heart from uremic rats [34–36]. Several groups investigating the mechanisms of growth impairment in renal failure have examined PTH/PTHrP receptor mRNA expression within the growth plate cartilage of uremic rats [37, 38] where downregulation of receptor mRNA expression was found to occur. Although histomorphometric data suggest that human bone cells exhibit PTH resistance in renal failure, there is no explanation for this at the cellular level *in vivo*. Cloning of the human PTH/PTHrP receptor gene [39] and the development of techniques such as *in situ* hybridization have made it possible to address this. Using *in situ* hybridization (for methodology see [40]), we have examined PTH/PTHrP receptor mRNA expression in human renal bone compared with normal, Pagetic, and healing fracture callus and found greatest expression over plump osteoblasts in areas of active bone formation (Fig. 1a, b). Although PTH stimulates bone resorption, *in vitro* studies have suggested this to be indirect, i.e., mediated via osteoblasts. However, in the three high turnover states actively resorbing osteoclasts were positive for PTH/PTHrP receptor mRNA (Fig. 1c, d) suggesting that osteoclasts may be capable of responding directly to PTH. Mean signal density of PTH/PTHrP receptor mRNA signal over osteoblasts in renal high turnover bone was only 37% of that found in non-renal high turnover ($P < 0.05$) and 49% of that found in normal bone ($P < 0.05$). Osteoblast PTH/PTHrP receptor mRNA signal in adynamic bone was 25% of normal bone ($P < 0.05$) and 51% of that found in renal high turnover bone ($P < 0.05$). These results demonstrate a downregulation of osteoblast PTH/PTHrP receptor mRNA in end-stage renal failure, in comparison to normal and nonrenal high turnover bone, and this may represent the molecular basis for resistance of skeletal tissue to PTH in uremic patients (unpublished data). No sig-

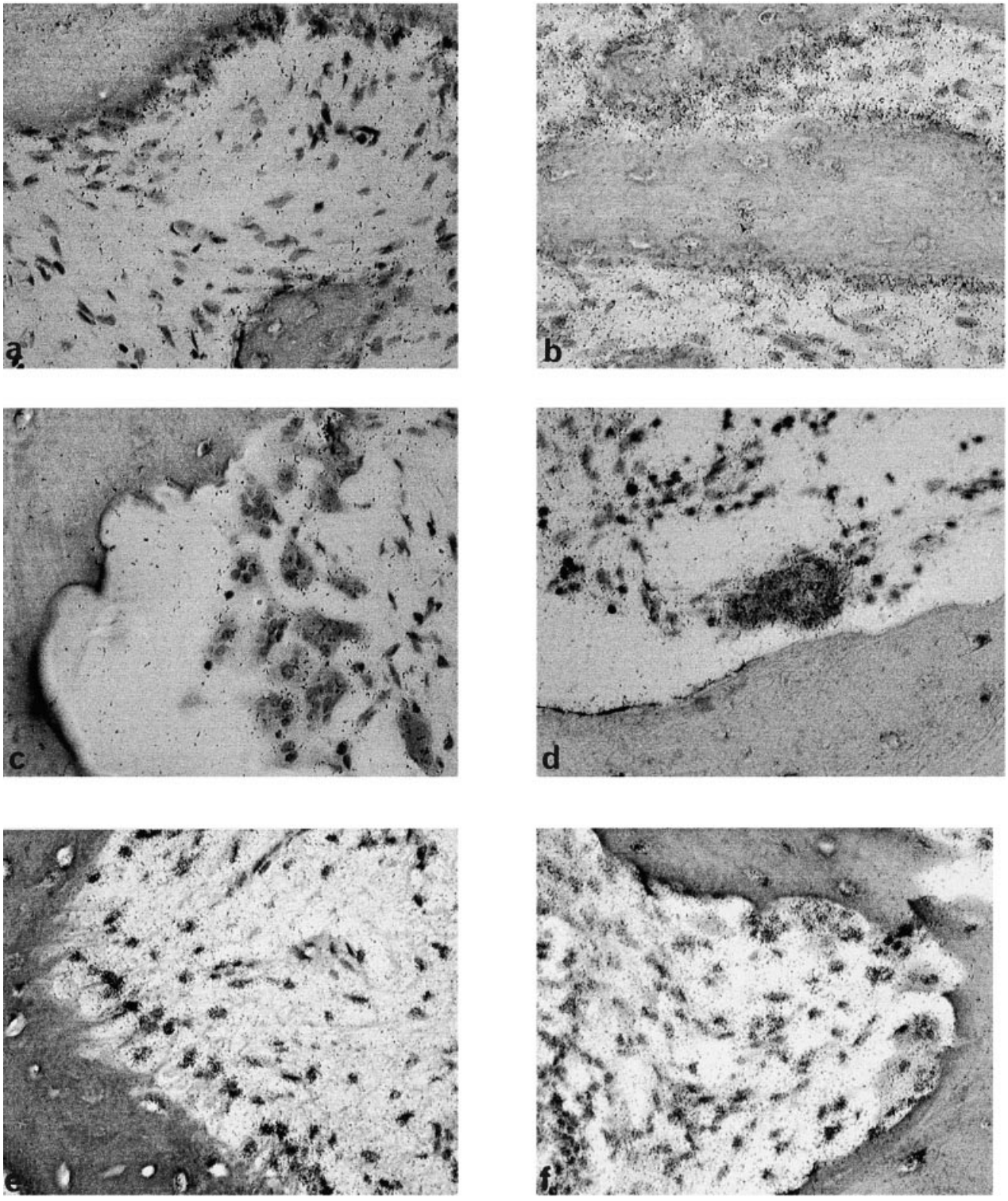

Fig. 1. PTH1R and TGF-β mRNA expression. Expression of PTH1R mRNA by osteoblasts in (*a*) renal hyperparathyroid bone and (*b*) in normal fracture callus. Note the increase in signal seen in nonuremic osteoblasts compared to those in renal bone. (*c*) PTH1R mRNA expression by osteoclasts in hyperparathyroid bone. (*d*) PTH1R mRNA expression by osteoclasts in pagetic bone. (*e*) TGF-β mRNA expression in renal hyperparathyroid bone in osteoblasts. (*f*) TGF-β mRNA expression in renal hyperparathyroid bone in osteoclasts.

nificant correlations were found between PTH/PTHrP receptor mRNA and serum PTH, ionized calcium, serum phosphate, serum vitamin D_3, age at time of biopsy, and duration of dialysis, thus making it difficult to draw any definite conclusions about the role of these factors in PTH/PTHrP receptor mRNA modulation. However, the finding of PTH receptor downregulation in adynamic bone despite levels of PTH within or just above the normal range suggests that PTH *per se* is not the key factor in downregulation. This is supported by data from Urena and colleagues who have shown that thyroparathyroidectomy does not prevent renal PTH/PTHrP receptor mRNA downregulation in uremic rats [33] and also from the absence of a correlation between serum PTH level and reduced receptor mRNA of uremic rat growth plate chondrocytes [38].

GROWTH FACTORS, BONE REMODELING, AND RENAL OSTEODYSTROPHY

In studies on the molecular mechanisms, which contribute to changes in bone formation seen in patients with renal failure, investigations have focussed on the role of circulating and locally produced cytokines and growth factors. Such studies suggest that alterations in the expression of locally acting growth factors and cytokines may be important in explaining the altered bone remodeling in renal osteodystrophy. Interleukin-6 (IL-6) and IL-6 receptor (IL-6R) mRNA is expressed in osteoblasts, osteocytes, osteoclasts and bone marrow cells in patients with hyperparathyroidism [41]. Semiquantitative analysis of hybridization signal indicated that activity of osteoclasts, as assessed by erosion depths of resorbing lacunae, is paralleled by IL-6R mRNA expression, suggesting that IL-6 and IL-6R are intricately involved in osteoclastic bone resorption in renal osteodystrophy. Of the various bone growth factors produced by osteoblasts and regulated by PTH, transforming growth factor β (TGF-β) and insulin-like growth factors (IGF-I and -II) may be important in the altered bone remodeling in renal osteodystrophy.

TGF-β

TGF-β is one of the most abundant growth factors in bone [42, 43]. Synthesized by osteoblasts and osteoclasts, and stored in the bone matrix, its effects are complex but in general appear to promote bone formation and inhibit bone resorption. *In vitro* studies show that TGF-β affects osteoblasts at all stages of the remodeling cycle. In general, TGF-β has potent chemotactic properties and has been shown to promote osteoblast recruitment to sites of new bone formation [44]. The primary effect on osteoblasts however, is to promote differentiation to mature matrix producing cells stimulating synthesis and secretion of matrix proteins (type I collagen, osteopontin, and decorin). The major effect on osteoclasts is inhibitory, preventing recruitment and activation of osteoclasts [45] and inducing apoptosis of mature cells [46]. TGF-β expression is regulated in a complex manner by several factors including PTH. PTH increases TGF-β production in cultures of normal human osteoblast-like cells [47]. Various studies have also shown that TGF-β may modulate the PTH/PTHrP receptor, although the exact effect (down or up regulation) appears to vary with the cell model used and the stage of cell differentiation [48–51].

Although the role of TGF-β has been extensively investigated in the development of glomerular disease [52], little is known about the role of TGF-β in the pathogenesis of renal osteodystrophy. Jiang et al [53] compared intraplatelet and plasma levels of TGF-β in hemodialysis patients with normal controls and found that patients with renal osteodystrophy had significantly higher levels than in patients without bone disease and concluded that renal osteodystrophy may stimulate overproduction of TGF-β in patients undergoing hemodialysis. Our *in situ* hybridization studies revealed that TGF-β mRNA was predominantly localized to osteoblasts, although some signal was observed over osteoclasts and osteocytes in renal hyperparathyroid bone (Fig. 1e, f). Semiquantitative analysis of hybridization signal showed a decrease in TGF-β mRNA in the renal bone samples. The lowest levels of expression were seen in adynamic osteoblasts with levels that were 56%, 39% and 28% of the expression seen in hyperparathyroid, normal and nonrenal high turnover osteoblasts, respectively. TGF-β mRNA expression by hyperparathyroid osteoblasts was 70% and 50% of that seen in normal and nonrenal high turnover osteoblasts. Significant correlations were found between TGF-β mRNA expression and serum PICP (a serum marker of bone formation) and the histomorphometric indices of bone formation rate, trabecular apposition rate, and mineralizing surfaces. This is consistent with many *in vitro* studies showing that TGF-β stimulates synthesis of bone matrix proteins.

IGF-I

IGF-I and -II are anabolic peptides that are structurally and functionally related to insulin. IGF-I is a mediator of growth hormone action in various tissues including bone and is synthesized mainly by the liver but also by bone and cartilage cells [54]. IGFs are key regulators of bone formation, decreasing collagen degradation, increasing bone matrix deposition and increasing osteoblastic cell recruitment. The major skeletal hormones appear to be important in regulating the effects of IGF-I. Both *in vitro* and *in vivo* studies suggest that the anabolic effects of intermittent PTH are mediated through local increased IGF-I expression [55, 56]. The role of IGFs in renal osteodystrophy is unclear. However, there is

increasing evidence that individuals with renal failure have IGF-I resistance [57]. This resistance appears to be multifactorial, despite normal levels of IGF-I there is an elevation of IGFBP-1 which is likely to reduce IGF-I bioactivity [58]. There may also be an IGF-I receptor defect [59] and finally IGF-I production by a variety of tissues appears to be reduced in uremic animals, with a reduction in steady state mRNA levels in liver [60], skeletal muscle [59] and growth plate cartilage [61]. Using *in situ* hybridization (ISH) we have analyzed IGF-I expression in renal bone. And found expression predominantly in osteoblasts. Semiquantitative analysis of hybridization signal over osteoblasts showed that there was a decrease in IGF-I mRNA in renal bone compared to normal and nonrenal high turnover bone. Levels of signal were significantly lower in adynamic bone than in the hyperparathyroid bone. These findings suggest that there is downregulation of osteoblast IGF-I in renal bone. Comparison of IGF-I mRNA signal density with serum biochemical markers showed a significant correlation with intact PTH levels, consistent with the suggestion that PTH action may be mediated by IGF-I. The lower levels in adynamic bone may reflect the lower serum intact PTH levels in patients with adynamic bone. Interestingly, other workers have found lower levels of IGF-I mRNA expression by osteoblasts in older bone [62, 63] where similarities with adynamic bone exist.

The observed histologic patterns in renal osteodystrophy are the net result of complex interactions between numerous systemic and local factors. *In situ* molecular biology techniques such as ISH allow researchers to examine the abnormalities of renal osteodystrophy at bone cell level by analysis of gene expression. For example, using *in situ* hybridization we have shown that there is downregulation of PTH/PTHrP receptor mRNA *in vivo* in human renal osteodystrophy confirming the long-held suspicion that alteration of receptor regulation is likely to occur. It is likely that this observed receptor downregulation results in an "altered threshold" for cellular response to PTH, so that serum PTH levels approximately 2–4 times normal are required to maintain normal bone turnover. The finding of lower levels of TGF-β and IGF-I mRNA (which is likely to be mirrored by protein synthesis) suggests that an "inhibitory shift" in cellular responses to PTH does occur. Interestingly, in studies of osteopenia due to aging and unloading, decreased osteoblast synthesis of TGF-B and IGF-I was associated with defective osteoblast recruitment, which could explain the low bone cellular activity in adynamic bone. Such studies thus provide a valuable insight into the cellular mechanisms underlying renal osteodystrophy.

ACKNOWLEDGMENTS

We thank Dr. Philip Moore and Mrs Pauline Baird for their contribution to the work, and the Baxter Extramural Grant Program and Foundation for Nephrology for funding our studies.

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