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IL-1β, TNF-α, TGF-β, and *b*FGF expression in bone biopsies before and after parathyroidectomy

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IL-1β, TNF-α, TGF-β, and *b*FGF expression in bone biopsies before and after parathyroidectomy.

Background. There is growing evidence pointing to an involvement of cytokines and growth factors in renal osteodystrophy. In this study, the expression of interleukin-lβ (IL-1β), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), and *basic* fibroblast growth factor (*b*FGF) in bone biopsies taken from uremic patients before and 1 year after parathyroidectomy (PTX) was evaluated. Biochemical features and histomorphometric outcome were also studied.

Methods. Iliac bone biopsies were taken before and 1 year after PTX in nine uremic patients with severe hyperparathyroidism (HPT). Immunohistochemical techniques were used to identify the expression of IL-1β, TNF-α, TGF-β, and *b*FGF in these bone samples.

Results. At the time of the second bone biopsy, the mean serum total alkaline phosphatase activity was normal, whereas mean serum intact parathyroid hormone (iPTH) level was slightly above the upper limit of normal values. Histomorphometric analysis showed a decrease in resorption parameters and static bone formation parameters after PTX. Dynamically, mineral apposition rate (MAR) and mineralization surface (MS/BS) decreased significantly. There was a marked local expression of IL-1β, TNF-α, TGF-β, and *b*FGF in bone biopsies before PTX, particularly in fibrous tissue and resorption areas. One year after PTX, IL-1β decreased from 23.6 ± 7.5% to 9.9 ± 3.1%, TNF-α from 4.5 ± 1.5% to 0.7 ± 0.8%, TGF-β from 49.6 ± 9.8% to 15.2 ± 4.6%, and *b*FGF from 50.9 ± 12.7% to 12.9 ± 7.9% ($P < 0.001$). A significant correlation was documented between cytokines and growth factors expression in bone with iPTH levels before and after PTX ($P < 0.05$).

Conclusions. Based on these results, we suggest that IL-1β, TNF-α, TGF-β, and *b*FGF are involved in bone remodeling regulation, acting as local effectors, possibly under the control of PTH.

Renal osteodystrophy comprises a variety of abnormal bone remodeling processes that develops in patients with end-stage renal failure. The main abnormalities are closely linked to alterations in bone turnover. Uremic patients may suffer from high turnover bone disease with various degrees of mineralization defect or low turnover osteodystrophy. The pathogenesis of renal bone disease relies mainly on alterations of the parathyroid hormone (PTH)-vitamin D axis. In fact, the excessive PTH production observed in uremic patients can lead to the well-known secondary hyperparathyroidism state (HPT). However, only excess production of PTH alone does not completely explain the bone abnormalities found in secondary HPT. Recent evidences have introduced new insights in the interplay between PTH and cytokines involved in the regulation of bone cells [1, 2]. In fact, high circulating levels of cytokines and growth factors have been extensively documented in uremic patients [3]. The overproduction of these mediators may have an important impact on bone metabolism, regulating different stages of the bone remodeling cycle in patients with renal osteodystrophy [4, 5].

Interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) are potent stimulators of bone resorption, both in vitro and in vivo [6]. The increased IL-1 plasma levels found in dialysis patients [3] may influence bone resorption by promoting the differentiation of osteoclasts initiating sites of bone erosion [7]. IL-1 may also stimulate prostaglandin synthesis, which independently contributes to bone resorption [8]. On the other hand, IL-1 can inhibit bone formation, as demonstrated by in vitro experiments [9]. TNF-α, which has also been found to circulate at high levels in uremic patients [3], has effects on bone resorption similar to those of IL-1, but with less potency [10]. TNF-α stimulates the differentiation of osteoclast precursors into mature cells, enhancing osteoclast activity [11], and can also inhibit bone formation [12].

High concentrations of transforming growth factor-β (TGF-β) have been found in bone [13]. Besides the well-known effects of TGF-β as a fibrogenic mediator in other organs [14], TGF-β also exerts effects in bone cell metabolism. In this context, TGF-β has mainly anabolic

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actions, enhancing bone cell formation [15], decreasing resorptive activity, and also attracting osteoblasts to resorption lacunae, initiating bone formation [16]. Another strong anabolic mediator of bone cells is *basic* fibroblast growth factor (*b*FGF). When activated during bone resorption, *b*FGF is able to stimulate bone cell replication and collagenous matrix modulation [17] and also promotes neovascularization [18]. Based on these observations, abnormalities in cytokines and growth factors that act on bone are likely to be critical in the pathogenesis of renal bone disease in patients with end-stage renal failure.

The prolonged survival of patients maintained on dialysis treatment has resulted in a growing number of uremic patients suffering from severe HPT, characterized by a marked increase in bone turnover, increased bone resorption and formation. When medical treatment fails, surgical reduction of the parathyroid gland mass may become necessary [19]. Immediately after parathyroidectomy (PTX), intact plasma PTH (iPTH) declines abruptly and persists at low levels for 1 to 2 years after surgery [20]. Several reviews reporting the effects of PTX on plasma biochemistry and on bone structure have been published [21, 22]. However, the effects of decreased PTH on bone pathophysiology, as well as the local expression of cytokines and growth factors, on long-term skeletal survey have not been investigated.

In this study, a group of uremic patients with severe HPT submitted to PTX was evaluated. The expression of IL-1β, TNF-α, TGF-β, and *b*FGF in bone biopsies taken from these patients before and 1 year after PTX was analyzed. Biochemical features and histomorphometric outcome were also studied.

METHODS

Group studied

Nine chronically uremic patients maintained on long-term hemodialysis (mean, 47.4 ± 18.1 months) were included in the study. Seven patientes were refereed from various hospitals of São Paulo to our institution. Another two patients entered long-term dialysis at University of São Paulo. The patients included six females and three males, ages 15 to 48 years (mean, 34.9 ± 11.3 years). The underlying renal disease was divided into glomerular disease ($N = 6$), nephrosclerosis ($N = 2$), and unknown ($N = 1$). The patients had severe HPT with markedly elevated serum levels of iPTH, ranging from 341 to 2246 pg/mL (mean, 1394 ± 556 pg/mL), and elevated alkaline phosphatase activity (AP) (1803 ± 898 U). After informed consent was obtained, the patients were submitted to an iliac crest bone biopsy that corroborated the severe HPT, including marrow fibrosis, accumulation of woven bone and osteoid, increased number and activity of osteoblasts, expansion of osteoid surfaces, and numerous osteoclasts and resorptive surfaces. Bone biopsies obtained from healthy skeletal of patients submitted to surgical orthopedic composed the control group. Control bone biopsies were examined with immunohistochemistry techniques. In addition, calcium, phosphate, AP activity, and iPTH were determinate in the serum samples of these patients.

The patients were unresponsive to clinical treatment, including vitamin D therapy, or had uncontrolled hyperphosphatemia despite the use of phosphate restriction and phosphate-binding antacids. Six patients received aluminum-containing phosphate binders for uncontrolled hyperphosphatemia. The other three patients received calcium-containing binders. Therefore, all patients were submitted to a total PTX, followed by heterotopic autotransplantation in the forearm. Thallium-technetium scans were performed before surgery for localization of glands.

After PTX, patients required high doses of calcium (0.27 ± 0.07 mg/kg/day) and calcitriol (0.045 ± 0.006 μg/kg/day). Then, calcium supplements were maintained in 87% of patients during the first 12 months of follow-up at 1.5 ± 0.23 g/day and calcitriol at 0.23 ± 0.12 μg/day in 74% of patients. These patients were reevaluated 1 year after PTX, with analyses of plasma calcium, phosphorus, AP, and iPTH. A second bone biopsy was also taken in each patient. Histomorphometric outcomes and immunohistochemical analysis, that is IL-1β, TNF-α, TGF-β, and *b*FGF expression, were analyzed in bone biopsies, comparing the samples before and 1 year after PTX. The Ethical Committee of the Hospital das Clinicas of University of São Paulo approved this study.

Biochemistry

Total serum calcium levels were measured by atomic absorption spectrophotometry (normal values, 8.8 to 10.5 mg/dL) and inorganic serum phosphate by the colorimetric method of Fisk-Subarrow (normal values, 2.8 to 4.8 mg/dL). Serum total AP activity was measured using panitro-*p*-phosphate as a substrate (normal values in adults, 50 to 190 U/L), and serum iPTH levels were determined by immunoradiometric assay of iPTH (Cis Bio-International, Gif-Sur-Yvette, France) with references values of 10 to 62 pg/mL.

Bone histology

Bone biopsies were obtained from a standard site near the anterior iliac crest using a Bordier trephine. Bone samples were fixed in 70% ethanol and processed for histomorphometry, as reported by Malluche and Faugere [23]. Prior to biopsy, all patients were prelabeled with oral tetracycline (20 mg/kg/day, during 3 days), administrated in two time-spaced doses, separated by an interval of 10 days.

Morphometric data were obtained from 5 μm thick

sections of undecalcified toluidine-blue-stained sections with a semiautomatic image analyzer Osteomeasure (Osteometrics, Inc., Atlanta, GA, USA). At least two nonconsecutive sections were examined for each sample. All histomorphometric indices were reported according to the nomenclature recommended by the American Society of Bone and Mineral Research (ASBMR) [24].

Static bone histology included bone volume and fibrosis as a percentage of tissue volume, osteoid volume as a percentage of bone volume, and osteoid thickness in micrometers. Osteoid surface, osteoblast surface, osteoclast surface, and aluminum surface were expressed as percentages of total trabecular surface. Trabecular number and trabecular separation were expressed in micrometers.

Dynamic bone parameters were obtained on unstained sections examined by fluorescence microscopy. The mineral apposition rate (MAR) was determined from the distance between the two tetracycline labels, divided by the time interval between the administrations of the two labels and expressed in micrometers per day. The percentage of double tetracycline-labeled surface of total trabecular surface (MS/BS) and bone formation rate (BFR) completed the dynamic evaluation. As normal control for Brazilian subjects was not available, the results were compared to normal values reported for European subjects by Bordier, Zingraff, Gueris [25], Meunier et al [26], and Charhon et al [27].

Immunohistochemical method

Immunostaining for IL-1β, TNF-α, TGF-β and *b*FGF was done with the avidin-biotin peroxidase method [28, 29] on plastic embedded bone marrow iliac crest biopsies [30].

The plastic mounted on the slides of sections was removed by immersing them in acetone (Merck, Darmstadt, Germany) for 12 hours at room temperature. Bone sections were rehydrated in graded alcohol solutions. To block endogenous peroxidase, the hydrated specimens were transferred to 3% hydrogen peroxide in 70% methanol for 90 minutes. Prior to the immunostaining procedure, the sections were incubated with 5% normal serum from the same species of the secondary antibody to avoid nonspecific staining. The samples were then incubated with avidin-biotin solutions to block nonspecific binding of these compounds. Sections were incubated overnight at 4°C in a humidified chamber using the primary antibodies: monoclonal mouse antihuman IL-1β (R&D Systems, Minneapolis, MN, USA), polyclonal goat antihuman TNF-α (R&D Systems), polyclonal rabbit antihuman TGF-β (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and polyclonal rabbit antihuman *b*FGF (Santa Cruz Biotechnology).

After incubation with the primary antibody, sections were rinsed in phosphate-buffered saline (PBS) buffer and then incubated with biotinylated secondary antibody: horse antimouse (Vector, Burlingame, CA, USA) to identify IL-1β, horse antigoat for TNF-α, goat antirabbit (Vector) to identify TGF-β and *b*FGF. After rinsing in PBS buffer, the slides were incubated with avidin-biotin peroxidase complex (ABC-kit, Vector). The slides were then developed using the diaminobenzidine (DAB) substrate (Vector). The sections were rinsed in distilled water and counterstained with haemalaum (Merck, Darmstadt, Germany).

Negative controls were carried out simultaneously with all experiments by omitting incubation with primary antibodies. For additional negative controls, sections were incubated with an irrelevant human immunoglobulin (IgG) (Sigma Chemical Co., St. Louis, MO, USA). In addition, sections were also incubated with mouse monoclonal anti-CD68 (Dako, Co., Glostrup, Denmark) for a positive control.

Table 1. Serum biochemical parameters before and 1 year after parathyroidectomy (PTX)

	Before PTX	After PTX	Control
Serum calcium *mg%*	8.5 ± 2.9	9.4 ± 0.8	9.3 ± 1.1
Serum phosphorus *mg%*	7.0 ± 1.3[a]	5.7 ± 1.2[a,b]	3.8 ± 0.6
Alkaline phosphatase *U/L*	1803 ± 898[a]	105 ± 43[b]	81.3 ± 19.8
iPTH *pg/mL*	1394 ± 556[a]	70 ± 97[a,b]	19.5 ± 8.5

[a] $P < 0.05$ vs. control
[b] $P < 0.05$ vs. before PTX

Quantitative analysis of cytokines and growth factors expression

The degree of IL-1β, TNF-α, TGF-β, and *b*FGF expression in bone biopsies was measured by the point-counting technique [31] at a magnification of ×100, using a 176-point ocular grid. Counting was performed on 25 microscopic fields for each bone sample. Each point was counted as either "positive" or "negative." The area of immunopositivity in the tissue was determined by point counting, determined as the number of positive points in the tissue compared to the total number of points. For this study, two physicians evaluated all slides in a blinded fashion without knowledge of the cases. The results were analyzed as a percentage of total tissue.

Statistical analysis

Results are presented as mean ± SD. ANOVA one-way test and Tukey post-test were used to compare the groups (before PTX, after PTX, and control group). Spearman's correlation coefficient r was used for different correlation analysis.

RESULTS

Biochemical findings

The biochemical data are presented in Table 1. Prior to PTX, serum calcium levels were 8.5 ± 2.9 mg%. Phos-

Table 2. Static histomorphometric parameters before and one year after parathyroidectomy (PTX)

	BV/TV %	OV/BV %	O.Th *mm*	OS/BS %	Ob.S/BS %	Oc.S/BS %	Tb.Sp *mm*	Tb. N *mm*	FB.V %	Al.S/BS %
Before PTX	41.4 ± 3.8	25.0 ± 4.5	17.7 ± 1.5	65.9 ± 6.0	24.5 ± 4.0	2.2 ± 0.5	163.7 ± 25.8	4.0 ± 0.4	23.7 ± 4.2	16.7 ± 6.9
After PTX	27.0 ± 3.3	9.8 ± 4.3	12.0 ± 1.9	33.3 ± 10.2	2.7 ± 1.0	0.1 ± 0.0	382.5 ± 88.0	2.4 ± 0.3	0.5 ± 0.2	47.8 ± 13
	$P < 0.001$	$P < 0.05$	NS	$P < 0.05$	$P < 0.001$	$P < 0.001$	$P < 0.05$	$P < 0.001$	$P < 0.001$	$P < 0.05$

Abbreviations are: BV/TV, bone volume; OV/BV, osteoid volume; O.Th, osteoid thickness; OS/BS, osteoid surface; Ob.S/BS, osteoblast surface; Oc.S/BS, osteoclast surface; Tb.Sp, trabecular separation; Tb. N, trabecular number; FB.V, fibrosis; Al.S/BS, aluminum surface; NS, not significant.

Table 3. Dynamic histomorphometric parameters before and 1 year after parathyroidectomy (PTX)

	MAR *μm/day*	MS/BS %	BRF/BS $\mu m^3/\mu m^2/day$	MLT *days*
Before PTX	1.7 ± 0.2	18.1 ± 2.5	0.3 ± 0.1	41.2 ± 11.2
After PTX	0.9 ± 0.1	9.2 ± 4.2	0.1 ± 0.0	31.5 ± 23.7
	$P < 0.05$	$P < 0.05$	$P < 0.05$	NS

Abbreviations are: MAR, mineral apposition rate; MS/BS, mineralization surface; BRF/BS, bone rate formation; MLT, mineralization lag time; NS, not significant.

phorus was elevated in all patients of this study. Total serum AP and iPTH levels were substantially elevated in all patients.

One year after PTX, serum calcium was not significantly different, but serum phosphorus levels decreased significantly ($P < 0.05$). AP activity and iPTH dropped dramatically ($P < 0.001$).

Bone histomorphometry

Prior to PTX, bone histologic features were categorized into osteitis fibrosis ($N = 4$), characterized by markedly increased osteoclastic activity, eroded bone surfaces, marrow fibrosis, and no mineralization defects, and mixed lesions ($N = 5$), comprising elements of both HPT and osteomalacia [23].

Histomorphometric analysis of bone biopsies taken before PTX confirmed previous diagnosis of HPT. Biopsies showed an increase in resorption (osteoclast surface, 2.2 ± 0.5%) and static bone formation parameters (osteoid surface, 65.9 ± 6.0%, and osteoblast surface, 24.5 ± 4.0%) (Table 2). A high degree of fibrosis was identified in bone marrow (23.7 ± 4.2%), and aluminum deposits covered a mean of 16.7 ± 6.9% of trabecular surfaces.

Dynamically, the extent of bone surface that exhibited tetracycline fluorescence was increased (MAR, 1.7 ± 0.2 μm/day, and MS/BS, 18.1 ± 2.5%). Bone rate formation and mineralization lag time (MLT) were elevated (0.3 ± 0.1 $\mu m^3/\mu m^2$/day, and 41.2 ± 11.2 days, respectively), indicating increased bone formation rate and an absence of a mineralization defect (Table 3).

Biopsies performed 1 year after PTX disclosed a significant reduction in resorption parameters (osteoclast surface, 0.1 ± 0%; $P < 0.001$), and static bone formation parameters decreased to osteoid surface, 33.3 ± 10.2% ($P < 0.05$), and osteoblast surface, 2.7 ± 1.0% ($P < 0.001$). Therefore, the mean volume of empty space increased (trabecular separation = 382.5 ± 88.0 mm; $P < 0.05$), and the solid material significantly decreased (trabecular number = 2.4 ± 0.3 mm; $P < 0.001$). Fibrosis regressed in all patients (0.5 ± 0.2%; $P < 0.001$), but aluminum surface increased significantly to 47.8 ± 13% ($P < 0.05$).

Dynamically, MAR and MS/BS decreased significantly (0.9 ± 0.1 μm/day, and 9.2 ± 4.2 μm/day; $P < 0.05$, respectively). BFR decreased to 0.1 ± 0.0 $\mu m^3/\mu m^2$/day ($P < 0.05$), and MLT to 31.5 ± 23.7 days. Consequently, four patients developed aplastic lesions and two patients osteomalacia. Another two patients showed normal histology and one presented a mild lesion.

Immunohistochemistry on bone biopsies

Control bone biopsies from healthy skeletal showed almost no staining for the cytokines and growth factors studied. In contrast, bone biopsies obtained from patients with HPT before PTX presented intense positivity for IL-1β, particularly in resorption areas and in medullary fibrosis (Fig. 1A). In bone biopsies taken 1 year after PTX, only a discrete staining for IL-1β was detected, mainly in the remaining fibrous tissue (Fig. 1B). There was a significant reduction in IL-1β immunostaining in biopsies, from 23.6 ± 7.5% to 9.9 ± 3.1% after PTX ($P < 0.001$). Normal control biopsies showed weak immunostaining for IL-1β (0.70 ± 0.09%, $P < 0.001$ vs. before PTX; $P < 0.05$ after PTX) (Fig. 2).

TNF-α expression in biopsies taken from patients with secondary HPT was observed mainly in resorption lacunae, occupying 4.5 ± 1.5% of total bone tissue (Fig. 1C). Only weak stain for TNF-α was observed in bone biopsies 1 year after PTX (0.7 ± 0.8%), and a significant decrease in TNF-α expression was documented ($P < 0.001$) (Fig. 1D). In control biopsies, almost no immunostaining for TNF-α was observed (0.04 ± 0.03%; $P < 0.001$ vs. before PTX).

Figure 1E illustrates immunostaining for TGF-β in a bone biopsy performed before PTX in a patient with severe HPT. TGF-β expression was detected particularly in resorption lacunae and in the fibrotic marrow. In addition, some cells exhibited immunopositivity for TGF-β in the tissue adjacent to the fibrotic marrow. Total quanti-

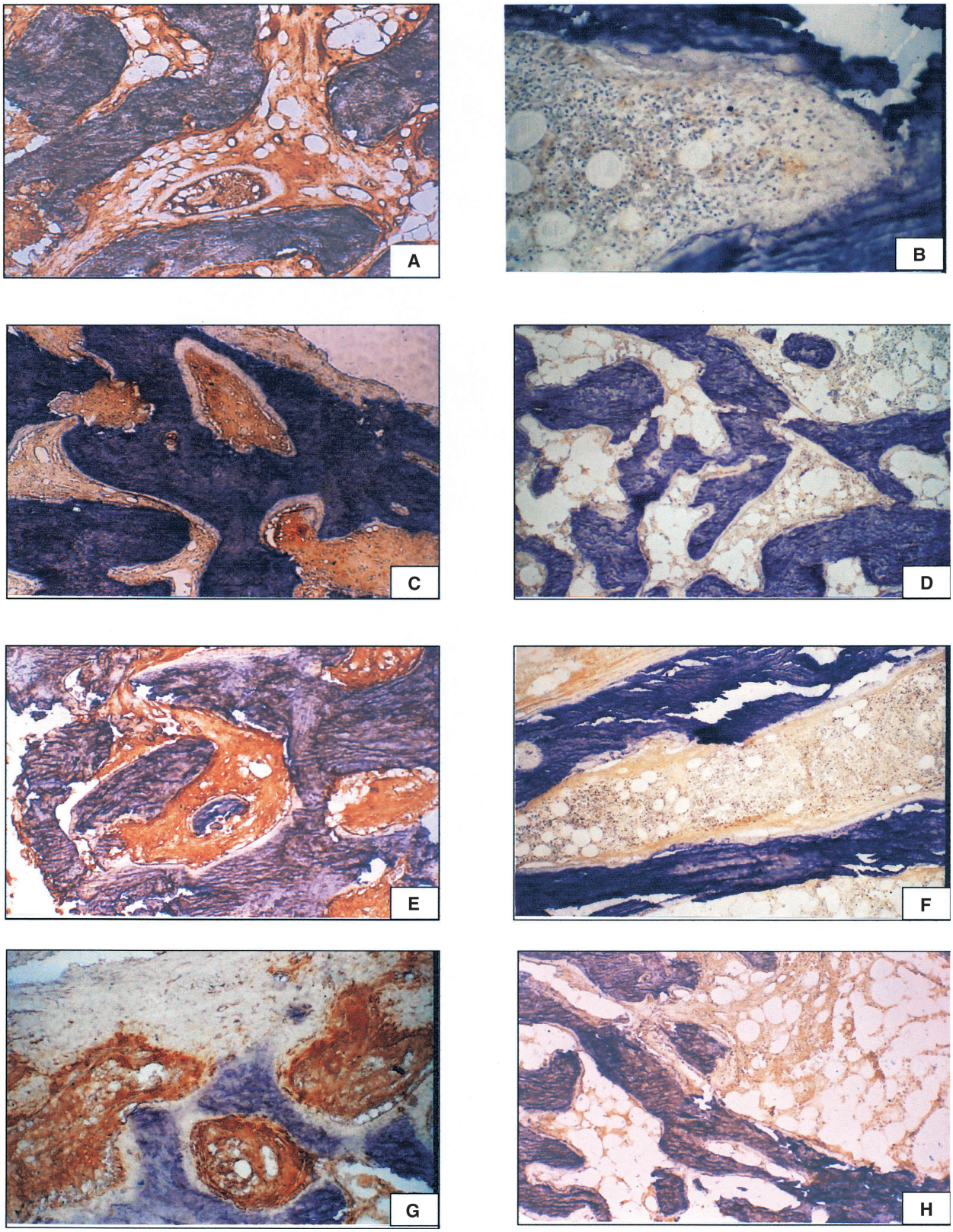

Fig. 1. Immunohistochemistry for cytokines and growth factors in bone biopsies. Positive staining appears as brown color (×100). (*A*) Interleukin-1β (IL-1β) expression in medullar fibrosis and resorption areas in a patient with severe hyperparathyroidism (HPT). (*B*) After parathyroidectomy (PTX), only a discrete positivity for IL-1β is observed. (*C*) Tumor necrosis factor-α (TNF-α) expression in resorptive lacunae before PTX. (*D*) Almost no TNF-α staining after PTX. (*E*) Intense immunostaining for transforming growth factor-β (TGF-β) in the fibrous area adjacent to the cellular tissue. (*F*) After PTX, a very weak immunostaining for TGF-β is observed. (*G*) *basic* fibroblast growth factor (*b*FGF) in the fibrotic marrow replacement of the normal bone marrow tissue before PTX. (*H*) Weak staining for *b*FGF in osteoid surface after PTX.

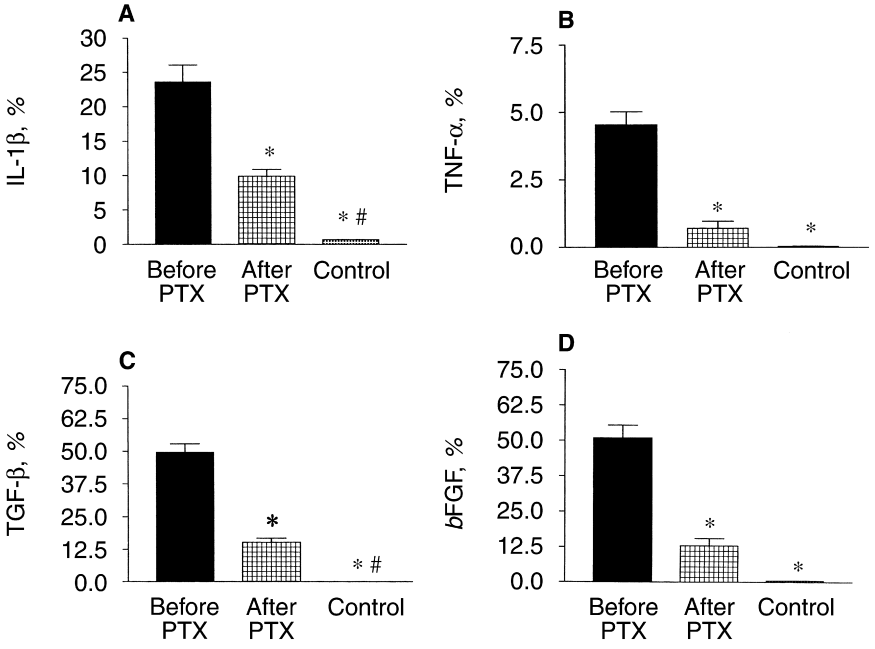

Fig. 2. Quantitative expression of interleukin-1β (IL-1β) (*A*), tumor necrosis factor-α (TNF-α) (*B*), transforming growth factor-β (TGF-β) (*C*), and *basic* fibroblast growth factor (*b*FGF) (*D*) (%) of cytokines and growth factors in bone biopsies before and 1 year after parathyroidectomy (PTX). $*P < 0.001$ vs. before PTX; $\#P < 0.05$ vs. after PTX.

tative staining counts for TGF-β before PTX was 49.6 ± 9.8% of the total area. After PTX, TGF-β expression was detected only in areas adjacent to the lining cells (Fig. 1F), occupying 15.2 ± 4.6% of the total area ($P <$ 0.001 vs. before PTX). Control group showed a weak staining for TGF-β, 0.05 ± 0.0% ($P < 0.001$ vs. before PTX and $P < 0.05$ vs. after PTX).

Bone biopsies of HPT performed before PTX showed a marked staining for *b*FGF (50.9 ± 12.7%) in the fibrotic marrow replacement of the normal bone marrow tissue (Fig. 1G). Only a faint staining for this growth factor appeared after PTX (12.9 ± 7.9%; $P < 0.001$) (Fig. 1H). In the control group, very weak immunostaining was detected (0.48 ± 0.09%; $P < 0.001$ vs. before PTX) (Fig. 2).

Due to the marked expression of these cytokines and growth factors observed in areas of bone marrow fibrosis, a correlation between pathologic fibrosis and expression of cytokines and growth factors before versus after PTX was evaluated. There was a significant positive correlation in the extent of fibrosis with IL-1β expression ($r = 0.82$, $P < 0.0001$). Significant correlations were also observed with TNF-α expression ($r = 0.65$, $P = 0.0038$), TGF-β expression ($r = 0.89$, $P < 0.0001$), and *b*FGF expression ($r = 0.83$, $P < 0.0001$).

The expression of IL-1β and TNF-α, cytokines with known effects on bone resorption, were correlated with osteoclast surface. The IL-1β and TNF-α expression showed significant correlation with osteoclast surface ($r = 0.67$, $P = 0.002$; and $r = 0.75$, $P = 0.0003$, respectively).

Cytokine and growth factor expression detected in bone biopsies also demonstrated a significant correlation with PTH circulating (iPTH), before and after PTX (Fig. 3). There was a significant correlation of IL-1β expression with iPTH levels ($r = 0.51$, $P = 0.0317$). TNF-α expression and iPTH also were significantly correlated ($r = 0.74$, $P = 0.0004$). The growth factors, TGF-β and *b*FGF, were significantly correlated with iPTH ($r = 0.56$, $P = 0.0150$; and $r = 0.68$, $P = 0.0021$, respectively).

DISCUSSION

The present study analyzed, by means of immunohistochemistry, the expression of IL-1β, TNF-α, TGF-β, and *b*FGF in bone biopsies of patients submitted to PTX, comparing the findings before and 1 year after surgical removal of parathyroid glands. Histomorphometric outcome and biochemical parameters were also evaluated.

Excessive PTH production in uremic patients is associated with an increased mass of parathyroid tissue [32]. The surgical correction of secondary HPT results in a dramatic reduction of PTH secretion, as observed in this study. A severe drop in AP followed this reduction in parathyroid activity, indicating decreased activity of osteoblasts, in face of reduced osteoclastic activity.

Histomorphometric analysis of bone biopsies taken before PTX confirmed the severity of the secondary HPT. One year after PTX, the regression of HPT bone lesions was complete in eight patients, only one bone sample still had mild lesions. This improvement in the bone is expected from the decrease of the well-known hyperresorptive effect of PTH and indicates that PTX clearly benefited patients who had histologic evidence in bone

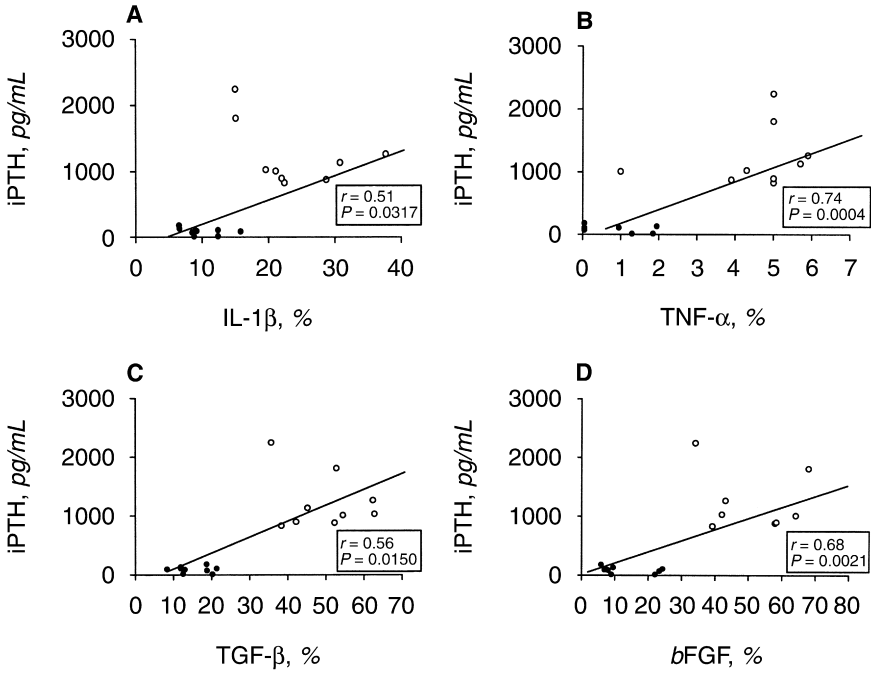

Fig. 3. Correlation between interleukin-1β (IL-1β) (*A*), tumor necrosis factor-α (TNF-α) (*B*), transforming growth factor-β (TGF-β) (*C*), and *basic* fibroblast growth factor (*b*FGF) (*D*) expression in bone biopsies with intact parathyroid (iPTH) levels before (○) and one year after (●) parathyroidectomy (PTX).

of HPT preoperatively. Bone marrow fibrosis regressed in all patients, corroborating the evidence for correction of severe bone lesions. However, osteomalacia or adynamic bone disease was reported in six patients, probably a result of an excessive reduction in PTH secretion, which can lead to inactive bone, so called "aplastic bone" [33]. In addition, PTX increased bone aluminum deposits, via dialysis water, probably due to favorable conditions for the development of low turnover osteomalacia.

Bone biopsies performed before PTX showed a marked staining for IL-1β, TNF-α, TGF-β, and *b*FGF. One year after PTX, less intense staining for these mediators was detected. IL-1β expression was intense in bone samples with HPT. Possible sources of IL-1β in bone are bone marrow cells, where, in fact, IL-1β was detected. Immunostaining of IL-1β was also observed in eroded surfaces of bone biopsies from patients with HPT, suggesting that bone cells are not only targets of IL-1β, but can additionally synthesize this cytokine. The local production of IL-1β suggests a pathogenic role of this mediator in the mechanism of bone remodeling in HPT.

IL-1β has been recognized as a potent bone-resorbing factor, biologic effects based mainly on its ability in stimulating osteoclast activity. Sabatini et al [34] demonstrated that infusion of IL-1β in normal mice increased the number of osteoclasts and increased bone resorption surfaces, causing hypercalcemia in these animals. On the other hand, blocking of IL-1β using antiserum completely inhibits bone-resorbing activity in cell cultures [35], and the use of soluble form of IL-1 receptor antagonists attenuates bone erosion in rats [36].

The analysis of TNF-α expression in bone biopsies disclosed similar results. Our immunohistochemical studies revealed marked expression of TNF-α in resorption lacunae in bone biopsies from patients with HPT. Similar to IL-1β, TNF-α stimulates bone resorption and inhibits bone formation [9], but with less potency than IL-1β [37]. The similar expression of IL-1β and TNF-α in bone biopsies and their similar biologic effects suggest that these cytokines act in synergism in the regulation of bone mass [6, 37].

In secondary HPT, overexpression of IL-1β and TNF-α may be a consequence of the uremic state, since high levels of these cytokines have been extensively demonstrated in plasma samples of dialysis patients [3, 38]. The marked down-regulation of IL-1β and TNF-α expression observed in bone biopsies performed 1 year after PTX, when plasma PTH activity reached normal levels, points to a role of PTH in regulating local cytokine production in bone tissue. A possible mechanism for bone resorption is via protein kinase C activation by PTH in osteoblasts. The actions of PTH on bone resorption seem to occur through osteoblast stimulation, which, in turn, produces osteoclastogenic factors, including cytokines. In this context, IL-1β, TNF-α, and IL-6 can be components of this signaling pathway, enhancing bone resorbing [39]. Thus, the actions of PTH on bone cell may occur through local cytokine activity at different stages of bone remodeling.

The development of bone marrow fibrosis and the growth regulation of bone cells may be mediated by local growth factors production [40]. The pathophysiology of marrow fibrosis in renal osteodystrophy has not been

completely elucidated. In secondary HPT, little is known about the factors that contribute to the development of marrow fibrosis. The extent of TGF-β deposition in bone biopsies in secondary HPT observed in the present study suggests an involvement of this growth factor in the fibrotic response. TGF-β can induce collagen synthesis and fibroblast proliferation, critical steps for the development of fibrosis [41]. In addition to the role of TGF-β in fibrogenesis, there is growing evidence that TGF-β has anabolic activity in bone metabolism. During the bone remodeling cycle, TGF-β decreases resorptive activity and attracts osteoblasts to resorptive lacunae, initiating bone formation [17]. Our results demonstrated a pronounced expression of TGF-β on osteoid surface, suggesting local production of this growth factor. In secondary HPT, these anabolic effects of TGF-β may be a compensatory response to accelerated bone resorption. A balance between resorption and bone formation is required in order to maintain mechanical integrity. TGF-β seems to play a role as a mediator of the anabolic effect of PTH on bone. In fact, Pfeilschfiter et al [42] observed that injections of PTH induced increased concentrations of TGF-β in rat bone leading to an increase in bone mineral density. The actions of TGF-β in bone could occur via a down-regulation of osteoblast PTH/PTH-related protein receptor (PTH/PTHrP), described on osteoblasts of uremic patients [43]. Jogen et al [44] demonstrated that TGF-β induces a decrease in steady state levels of PTH/PTHrP, resulting in decreased PTHrP receptor binding. Thus, TGF-β could induce a decreased response of osteoblasts to PTH stimulation, resulting in anabolic effects.

Similarly to TGF-β, the expression of *b*FGF was intense in bone biopsies before PTX, particularly in fibrous tissue of bone marrow and in the osteoid surface. *b*FGF is also a strong anabolic bone agent [45] and is produced by bone marrow stromal cells [46]. After bone resorption processes, latent factors, including *b*FGF, stimulate the repair component of the remodeling cycle and it may induce angiogenesis, contributing to bone formation [18]. Pun et al [45] demonstrated anabolic actions of FGF in rats after intravenous FGF administration. In our study, the effects of this growth factor were evidenced by a significant increase in bone formation indices. The exact mechanism of anabolic actions of *b*FGF is not completely understood, but it may exert a direct inhibitory action on osteoclast precursors [47]. The fibrogenic action of FGF is probably through activation of fibroblasts and matrix production. An important synergism between FGF and TGF-β acting on myofibroblast transdifferentiation could contribute to the pathogenesis of marrow fibrosis [48].

After PTX, the regression of medullar fibrosis and the decrease in remodeling sites were accompanied by a reduction in cytokine and growth factor expression. In addition to decreased production of PTH, a net suppressive effect of these local substances is possible, contributing to the low turnover status observed in bone biopsies. The markedly increased bone marrow fibrosis found in bone biopsies with severe HPT had a significant positive correlation with cytokine and growth factor expression. These observations suggest that the effects of increased PTH activity are mediated by local production of cytokines and growth factors. These local substances exert cell-to-cell communication, signalizing several functions in bone. At present, there is little information concerning the direct involvement of cytokines in the development of each stage of renal osteodystrophy. Advances in the recognition of disorders may lead to improved approaches to the prevention and treatment of bone disease. The possibility to assess various cytokines in bone cells and their bone microenvironment should allow us to understand the intricate mechanisms of renal osteodystrophy.

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