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Bone resorption and mRNA expression of IL-6 and IL-6 receptor in patients with renal osteodystrophy

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Bone resorption and mRNA expression of IL-6 and IL-6 receptor in patients with renal osteodystrophy. The cytokine interleukin-6 (IL-6) is a major cell regulatory factor that may play an important role in the bone remodeling of patients with renal failure. IL-6 exerts its action by binding to its receptor (IL-6R), which leads to transduction of a second messenger cascade within cells. *In vitro* as well as *in vivo* data point to IL-6 as an autocrine/paracrine factor in bone osteoclasts. Recently, bone cells from patients with Paget's disease were found to express IL-6 and IL-6R mRNA transcripts. However, in patients with renal bone disease, there is currently no *in vivo* evidence that osteoclasts have the capability to express mRNA for IL-6 and IL-6R. To investigate the potential expression of IL-6 and IL-6R in bone and its relationship to bone cell activity, iliac crest bone biopsies were performed in patients on chronic maintenance dialysis. Messenger RNA expression of IL-6 and IL-6R was studied using *in situ* hybridization histochemistry, and parameters of bone turnover were determined by bone histomorphometry. In the samples studied, mRNA expression of IL-6 and IL-6R was found in osteoclasts and bone marrow cells. Furthermore, we report the novel finding of increased IL-6R mRNA expression in osteoclasts engaged in increased bone resorption. The results of the present study suggest that the cytokine IL-6 is intricately involved in osteoclastic bone resorption and that expression of its receptor, IL-6R, in osteoclasts may parallel osteoclastic bone resorbing activity.

The cytokine interleukin-6 (IL-6) is postulated to play a significant role in bone remodeling by its ubiquitous presence in the bone microenvironment [1–4]. This cytokine exerts its cellular actions through its receptor (IL-6R), an 80 kDa ligand-binding protein, and a homodimer signal transducing glycoprotein 130 [5], triggering a second messenger cascade [6]. Osteoclast-progenitor cells as well as osteoblast-like cells *in vitro* can produce IL-6 [3, 7]. IL-6 can stimulate differentiation and proliferation of osteoclast precursor cells [3, 7, 8] and *in vivo* reports suggest that IL-6 may induce hypercalcemia, osteoclast formation, and subsequently, increased bone resorption [9]. Recent data demonstrated a direct stimulatory effect of IL-6 in osteoclasts' ability to produce cathepsin L, a cysteine proteinase indicative of the osteoclastic bone resorption process [10]. IL-6 induced dose-dependent increases of cathepsin L activity without affecting the overall number of osteoclasts.

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Although the functional role of IL-6 clearly points to its importance in bone remodeling, very little is known of the cellular localization in bone cells for IL-6 and IL-6R mRNAs *in vivo*. The only *in vivo* evidence of IL-6 and IL-6R expression, particularly in osteoclasts, is documented in patients with Paget's bone disease [11] suggesting that IL-6 may be an autoregulatory factor in these cells. There is currently no other molecular histologic evidence that this cytokine and/or its receptor are expressed in other metabolic bone diseases such as renal bone disease.

Renal osteodystrophy is a good model for the study of the relationship between IL-6, its receptor, IL-6R, and bone resorption. It is a disease that presents a wide range of bone remodeling abnormalities [2, 12]. Therefore, the present study was carried out to investigate the interaction between IL-6 and IL-6R mRNA expressions and bone resorption in iliac crest biopsies of patients with chronic renal failure.

Methods

Tissue sources and preparation

Bone samples were taken from seven patients that were on chronic maintenance dialysis using an electric drill (Straumann AG, Waldenburg, Switzerland). The patients were under local anesthesia during the procedure [13]. Biopsies from the anterior iliac crest were immediately fixed in fresh 4% paraformaldehyde at 4°C for 24 hours. Acetone dehydration, cold polymerization in methyl methacrylate (MMA), and plastic-embedding in MMA followed [14]. Serial sections 5 μm thick were cut with a Microm, model HM360 microtome (C. Zeiss, Thornwood, NY, USA).

For mineralized bone histology, sections were stained with the modified Masson-Goldner trichrome stain [15]. In addition, sections were stained with aurin tricarboxylic acid stain [16] and solochrome azurine [17] for detection of aluminum. Unstained serial sections were also prepared for phase contrast and fluorescent light microscopy.

In situ hybridization

Following plastic removal using 2-methoxyethyl acetate, tissue sections were deproteinated with proteinase K (0.1 μg/ml) for 15 minutes at 37°C to allow probe penetration, subsequently rinsed in 0.2% buffered glycine, washed in 0.1 M triethanolamine for five minutes, and acetylated for 15 minutes with the addition of 0.25%

acetic anhydride. The slides were then rinsed in 2× SSC and dehydrated through graded alcohols.

Hybridization riboprobe protocol. Riboprobes for human IL-6 and IL-6R mRNAs were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The IL-6 probe (ca 1.0 kb) was subcloned into the *Eco*RI site of pGEM7Zf and the T7 and SP6 RNA polymerases were utilized to generate the sense and anti-sense RNAs, respectively. The IL-6R probe (ca 1.2 kb) was subcloned into pBluescript KS. Sense and anti-sense RNAs were made by linearization with either *Eco*RI or *Bam*HI and using T7 or T3 RNA polymerases. The probes were generated in accordance with standard transcription protocols using MAXIscript *in vitro* transcription kit (Ambion Inc., Austin, TX, USA). Briefly, RNAse-free water, 10× transcription buffer, 200 mM DTT, 10 mM aliquots of ATP, CTP and GTP, and 12.5 U of RNAse inhibitor were combined with 1 μg of linearized DNA template, 10 U of appropriate polymerase and 40 μCi ^{35}S-UTP to a 20 μl volume. Transcription reaction occurred at 37°C for one hour followed by digestion of template with 2 U of DNAseI and ethanol precipitation with 5 M NH_4OAc, 100 mM EDTA, 100% EtOH, and 6 μg yeast tRNA at −70°C for one hour. Pellets were resuspended in sterile RNAse-free water. Specific activities of synthesized probes were determined to be 1.4×10^9 cpm/μg for IL-6 and 7.4×10^8 cpm/μg for IL-6R antisense riboprobes.

Fidelity of the probes was verified by 4% polyacrylamide gel electrophoresis of aliquots of respective probes. Controls for the experimental protocol included (1) tissue sections pretreated with RNAse A prior to proteinase K treatment and hybridization with antisense probes and (2) hybridization with sense-strand probes.

In situ hybridization histochemistry protocol. One million cpm of synthesized probe in 50 μl of hybridization buffer (Amresco, Solon, OH, USA), containing 50% formamide was applied to each slide. The diluted probe was placed on appropriately sized coverslips, which were subsequently applied to tissue sections. The slides were then placed in a chamber humidified with 50% formamide and incubated overnight at 50°C. The following day, coverslips were taken off in 2× SSC and unbound probe was digested in 100 μg/ml RNAse A. The slides were washed twice in 0.2× SSC (10 min/wash), incubated in 0.2× SSC at 65°C for one hour, then transferred into fresh 0.2× SSC. Dehydration through graded ethanols and air drying followed. The slides were subsequently prepared for X-ray and/or emulsion autoradiography with BioMax MR film or NTB2 emulsion (Kodak, Rochester, NY, USA).

Data analysis. The emulsion-dipped images were analyzed using a Zeiss Universal microscope (C. Zeiss, Thornwood, NY, USA) mounted with a Motion Analysis video camera, a Power Macintosh 8500 AV computer, and the NIH image software program. Histologic optical fields at 125× magnification were examined. The minimum number of osteoclasts assessed was determined by evaluating an increasing number of osteoclasts ranging from 20 to 100 cells. It was found that more than 50 osteoclasts did not change the mean and SD of the labeling intensity. Therefore, a minimum of 50 osteoclasts were analyzed in each sample. To qualitatively analyze the hybridization labeling density of the respective mRNAs, discrete cell bodies were scored on a graded scale. The grading criteria were: + for < 10 grains/cell; ++ for 10 to 50 grains/cell; +++ for 50 to 80 grains/cell; ++++ for grains confluent.

Bone histomorphometry

Static and dynamic parameters of bone structure, formation, and resorption were measured with the Osteoplan System II (Kontron, Munich, Germany) [18]. A minimum of 50 optical fields was evaluated at a magnification of 200× through the use of an objective with a numerical aperture of 0.4 using a Universal microscope (C. Zeiss, Thornwood, NY, USA). Erosion depth, which reflects osteoclast activity was calculated using the rectangular model from the area and length of the erosion cavity obtained by reconstruction [19-21]. All parameters comply with the nomenclature published by the histomorphometry nomenclature committee of the American Society of Bone and Mineral Research [22].

Measurement of serum parathyroid hormone (PTH) levels

Concentrations of serum PTH were determined in duplicate by the two-site immunoradiometric assay (IRMA) for intact PTH [23] using the Allegro™ Intact PTH assay kit (Nichols Institute, San Juan Capistrano, CA, USA). The interassay variation was 4%.

Statistical analysis

Results are expressed as mean ± SEM. Bivariate correlations were carried out using the Spearman rank test.

Results

Among the seven patients studied, there were four men and three women with a mean age of 48.8 ± 7.2 years (range 23 to 72 years) on chronic maintenance dialysis for a duration of 2.5 ± 0.6 years (range 10 months to 5 years). All patients had serum PTH levels above the normal range of 65 pg/ml (Table 1). Mineralized bone histology showed predominant hyperparathyroidism in three patients and mixed uremic osteodystrophy (such as association of secondary hyperparathyroidism with various degrees of a mineralization defect) in four patients (Table 1). None of the patients exhibited stainable aluminum at the bone-osteoid interface. The parameters of bone erosion are shown in Table 1. Serum PTH levels correlated with parameters of bone resorption ($r = 0.84$–0.94, $P < 0.02$).

IL-6 and IL-6R mRNA expression

All osteoclasts in bone tissue sections contained positive hybridization signals for IL-6 and IL-6R mRNAs (Fig. 1). The positive signal for IL-6 mRNA was easily observed, and osteoclasts contained 20 to 50 grains per cell (Fig. 1F). However, no marked difference was observed in the labeling intensity for IL-6 mRNA (Table 1).

In contrast to IL-6 mRNA labeling intensity, the hybridization signals for IL-6R mRNA in osteoclasts differed markedly from patient to patient (Table 1). The intensities for the IL-6R mRNA signal ranged from more heavily labeled cells containing up to 80 grains per cells to intensely labeled cells that exhibited the strongest hybridization signal (Table 1, Fig. 1 C, E).

The labeling intensities of IL-6R mRNA were commensurate with the parameters of bone resorption (Table 1). The most intense hybridization signals for IL-6R mRNA in osteoclasts were observed in the patients with the highest bone resorption, characterized by dissective and tunneling resorption sites (Fig. 1E). Also, within the same patient, osteoclasts found at the tip of

Table 1. Serum parathyroid hormone (PTH) levels, parameters of bone resorption, IL-6 and IL-6R mRNA signal intensities in seven patients with renal osteodystrophy

Patients	Serum PTH *pg/ml*	Diagnosis[a]	Erosion/ bone surface *%*	Osteoclast number/bone *#/100 mm*	Erosion depth *μm*	IL-6 mRNA[b]	IL-6R mRNA[b]
1	380	MUO	4.8	73	24.2	++	+
2	234	MUO	6.4	88	20.9	++	+
3	262	MUO	7.3	106	21.5	++	++
4	421	MUO	10.6	164	17.9	++	++
5	874	PHPT	13.0	214	35.2	++	++++
6	1007	PHPT	14.6	219	43.7	++	++++
7	1234	PHPT	25.9	281	33	++	+++

[a] Abbreviations used: MUO, mixed uremic osteodystrophy; PHPT, predominant hyperparathyroid; IL-6, interleukin-6; IL-6R, interleukin-6 receptor.
[b] Criteria for grading mRNA signal levels were as follows: + <10 grains/cell; ++ 10 to 50 grains/cell; +++ 50 to 80 grains/cell; ++++, grains confluent.

cutting cones of deep dissective lacunae exhibited stronger hybridization signal (Fig. 1E) than those in shallow surface resorption sites (Fig. 1C).

The bone marrow cells also contained positive hybridization signals for IL-6 and IL-6R mRNAs (Fig. 2 A, B). The signal varied but did not approach the intensity of IL-6R mRNA signal in osteoclasts of deep dissective resorption lacunae. Osteoblasts and osteocytes exhibited weak labeling which did not exceed the background signal level. Control experiments confirmed the specificity of probes. Bone tissue sections serially processed that were either probed with sense-strand riboprobes or pretreated with RNAse A did not contain positive hybridization signals for IL-6 or IL-6R mRNAs in specific bone tissue cells (Fig. 1 B, D).

Discussion

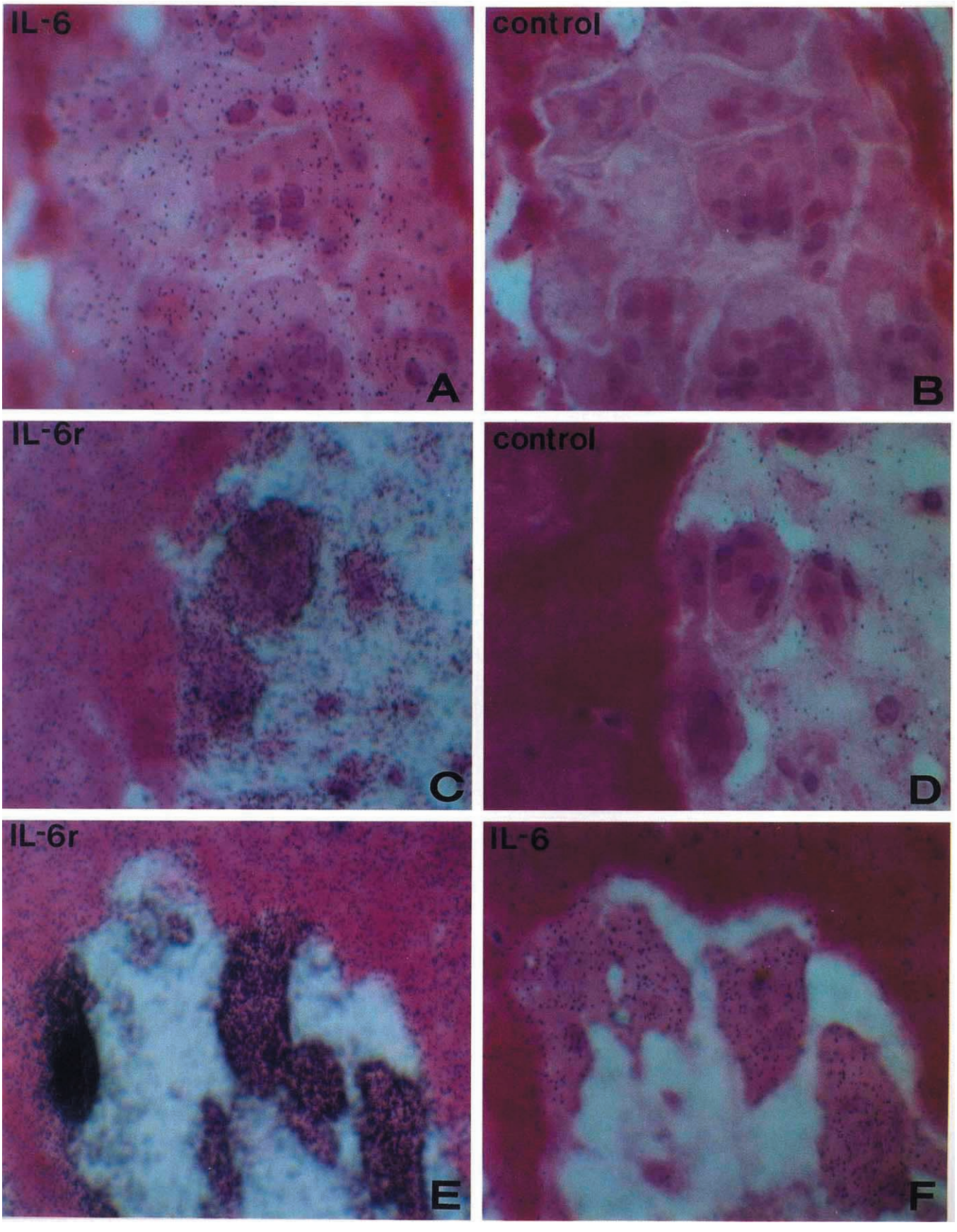
This report is in keeping with *in vitro* studies that demonstrated the presence of IL-6 in multinucleated cells [3, 7, 8, 24–26]. It also supports and further expands another *in vivo* report that mRNA transcripts for IL-6 and IL-6R are expressed in bone cells of patients with Paget's disease [11]. To our knowledge, the present study provides the first molecular histologic evidence that mRNAs for IL-6 and IL-6R are expressed in osteoclasts from undecalcified human bone tissue of patients with renal failure. In contrast to the findings from patients with Paget's disease, our study demonstrates localization of IL-6 and IL-6R mRNAs in patients with renal failure who should not have the potential problem of virally infected osteoclasts. Thus, the present observation should represent a more physiologic example of *in vivo* osteoclastic expressions of these mRNA transcripts. Together with data from patients with Paget's disease, the present study raises the possibility that in different bone diseases, local factors, including cytokines and their receptors, exert their contribution to bone remodeling by regulation of specific mRNA expression.

Our observation that osteoclasts have high levels of IL-6 mRNA expression confirms an earlier report noting that osteoclasts express significantly more IL-6 than osteoblasts [24]. The present study did not detect significantly increased IL-6 and IL-6R mRNA transcripts in osteoblasts, in contrast to the highly expressive osteoclasts and bone marrow stromal cells. This observation is in keeping with the *in vitro* data demonstrating that IL-6 does not elicit cellular response from osteoblast-like cells [27]. However, it may be that low detectable IL-6 trancripts may be one of the characteristics manifested in osteoblastic insufficiency described in renal failure patients [2, 28]. Thus, it is possible that the mRNA levels of IL-6 and IL-6R in osteoblasts may be too low to be detected by our methodology in patients with renal failure since osteoblasts of Pagetic bone appear to express these mRNA species [11].

It is of interest that we observed positive labeling for IL-6 and IL-6R mRNAs in bone marrow cells. It is clear that the bone marrow gives rise to the progenitor cells for both osteoclasts and osteoblasts [29]. *In vitro* studies indicate that these cells exhibit an extensive overlap in their phenotypic characteristics including expression of alkaline phosphatase and collagen type I [30, 31] and secretion of IL-6 and other cytokines [32, 33]. Evidence from animal models suggests that the bone marrow undergoes significant cellular changes in response to cessation of ovarian function and as a result of senescence [34, 35]. These studies lend credence to our observation in the bone microenvironment documenting IL-6 mRNA expression in bone marrow cells. However, the IL-6 and IL-6R mRNA expression in marrow cells requires further investigations that are beyond the scope of this report. Our observation that IL-6 and IL-6R are expressed in bone marrow cells suggests that this cytokine may play an autoregulatory function.

IL-6 is a multifunctional cytokine that has been shown to have profound effects on bone [1–4]. The bone loss associated with ovariectomy in mice has been shown to be prevented by administration of an antibody to IL-6, linking it with the effects of gonadal steroids such as estrogen on bone [36]. The novel finding in our study of increased IL-6R mRNA expression in osteoclasts suggests an interaction between these osteoclasts and bone remodeling, particularly in patients with high bone resorption. Moreover, the cytokine IL-6 may be intimately involved in bone resorption, potentially with osteoclast activation and, more importantly, expression of its receptor in osteoclasts appear to parallel the levels of osteoclastic bone resorbing activity.

Although IL-6R mRNA expression in osteoclasts correlated well with PTH levels, it is still not known whether PTH has a direct effect on osteoclasts. Although recent *in vitro* and *in vivo* data indicate that high PTH levels are associated with increased production of IL-6 and IL-6R [27, 37, 38], PTH receptors have yet to be detected in bone osteoclasts. Our present data support the hypothesis that high PTH levels in patients with renal failure may stimulate IL-6 and IL-6R mRNAs in osteoclasts but not osteoblasts. Thus, PTH could modulate osteoclastic activity through other factors synthesized and secreted by osteoblasts and marrow cells, including other cytokines and growth factors. It is well


IL-6
A
control
B
IL-6r
C
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IL-6r
E
IL-6
F

Fig. 1. *Brightfield photomicrographs of in situ hybridization for IL-6 and IL-6R mRNAs in osteoclasts of bone sections (thickness 5 μm).* Original magnification ×98; H and E counterstains. **A.** Osteoclasts in a resorption lacunae with positive hybridization signals for IL-6 mRNA. **B.** Serial section of bone shown in A processed with sense-strand probe as control. No labeling was observed in osteoclasts. **C.** Positive hybridization signal for IL-6R mRNA in osteoclasts of a surface resorption site. **D.** Serial section of bone shown in C processed through RNAse A pretreatment prior to hybridization with antisense probe. No labeling was observed in osteoclasts. **E.** Osteoclasts at the tip of a dissective cutting cone from the same patient sampled in C. Stronger hybridization signal was observed for IL-6R mRNA in osteoclasts (compared to C) of this more active resorptive site. **F.** Serial section of bone shown in E with positive hybridization signal for IL-6 mRNA in osteoclasts. The IL-6 mRNA signal appear less intense than the IL-6R mRNA signals in E. Publication of this figure in color was made possible by a grant from Dialysis Clinics Incorporated, Lexington, Kentucky, USA.

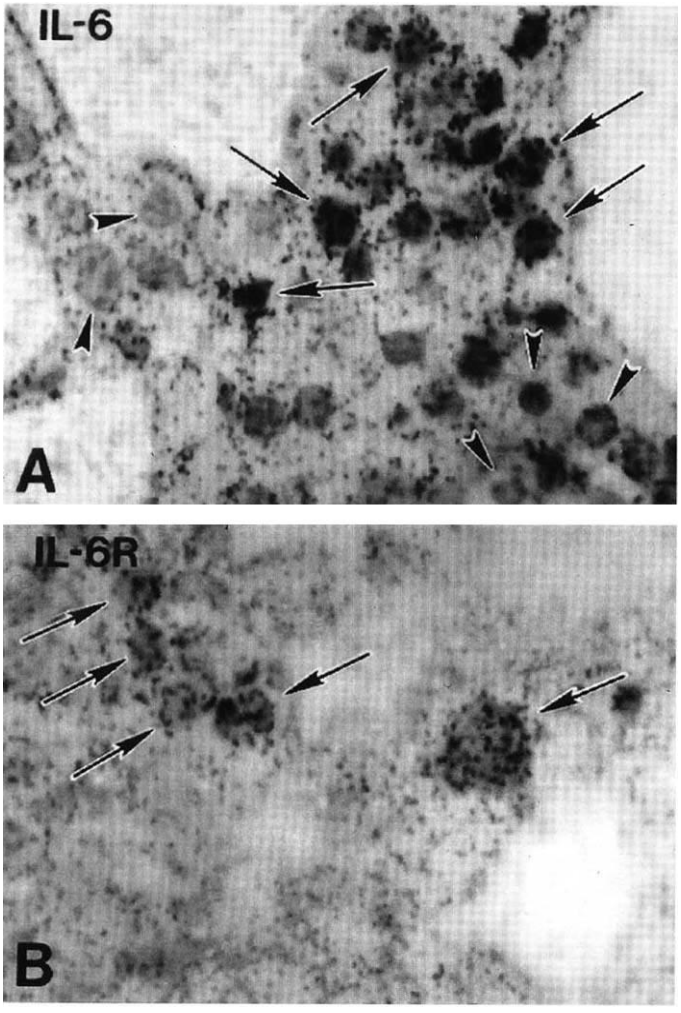

Fig. 2. *Brightfield photomicrographs of in situ hybridization for IL-6 and IL-6R mRNAs in bone marrow cells (thickness 5 μm).* Original magnification ×98; H and E counterstains. **A.** Positive hybridization signals for IL-6 mRNA in bone marrow cells (arrows). No labeling is present in cells denoted by arrowheads. **B.** Bone marrow cells (arrows) contained positive hybridization signals for IL-6R mRNA.

known that most factors that regulate bone resorption, including PTH, calcitriol, tumor necrosis factor-alpha and -beta, are believed to act indirectly on osteoclasts through effects on osteoblasts [39–45]. Investigations of the relationship between PTH and IL-6 and IL-6R in patients with renal failure may provide additional information on the extent of the contribution of PTH to the augmented expression of IL-6R mRNA in osteoclasts. This could be achieved by identification of cells that respond to PTH with secretion of osteoclast activity stimulatory factors. To our knowledge, this report provides the first evidence that the immunomodulatory cytokine, IL-6, and its receptor IL-6R, are associated with bone resorbing activity of osteoclasts. Continuing studies to identify cells that produce locally secreted cytokines and their pattern of expression in patients with renal failure are presently underway.

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