

## **Synovium as a source of increased amino-terminal parathyroid hormone-related protein expression in rheumatoid arthritis. A possible role for locally produced parathyroid hormone-related protein in the pathogenesis of rheumatoid arthritis.**

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### Research Article

Proinflammatory cytokines, including tumor necrosis factor (TNF) and interleukin 1 (IL-1), mediate the joint destruction that characterizes rheumatoid arthritis (RA). Previous studies have shown that parathyroid hormone-related protein (PTHrP) is a member of the cascade of proinflammatory cytokines induced in parenchymal organs during lethal endotoxemia. To test the hypothesis that NH<sub>2</sub>-terminal PTHrP, a potent bone resorbing agent, could also be a member of the synovial cascade of tissue-destructive cytokines whose expression is induced in RA, PTHrP expression was examined in synovium and synoviocytes obtained from patients with RA and osteoarthritis (OA). PTHrP production, as determined by measurement of immunoreactive PTHrP(1-86) in tissue explant supernatants, was increased 10-fold in RA versus OA synovial tissue. Synovial lining cells and fibroblast-like cells within the pannus expressed both PTHrP and the PTH/PTHrP receptor, findings that were confirmed by in vitro studies of cultured synoviocytes. TNF-alpha and IL-1beta stimulated PTHrP expression in synoviocytes, while dexamethasone and interferon-gamma, agents with some therapeutic efficacy in the treatment of RA, inhibited PTHrP release. Treatment of synoviocytes with PTHrP(1-34) stimulated IL-6 secretion. These results suggest that proinflammatory cytokine-stimulated production of NH<sub>2</sub>-terminal PTHrP by synovial tissue directly invading cartilage and bone in RA may mediate joint destruction through direct effects on cartilage or bone, or, indirectly, via the induction of mediators of bone resorption in the tumor-like synovium.

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# Synovium as a Source of Increased Amino-terminal Parathyroid Hormone-related Protein Expression in Rheumatoid Arthritis

## A Possible Role for Locally Produced Parathyroid Hormone-related Protein in the Pathogenesis of Rheumatoid Arthritis

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### Abstract

Proinflammatory cytokines, including tumor necrosis factor (TNF) and interleukin 1 (IL-1), mediate the joint destruction that characterizes rheumatoid arthritis (RA). Previous studies have shown that parathyroid hormone-related protein (PTHrP) is a member of the cascade of proinflammatory cytokines induced in parenchymal organs during lethal endotoxemia. To test the hypothesis that NH<sub>2</sub>-terminal PTHrP, a potent bone resorbing agent, could also be a member of the synovial cascade of tissue-destructive cytokines whose expression is induced in RA, PTHrP expression was examined in synovium and synoviocytes obtained from patients with RA and osteoarthritis (OA). PTHrP production, as determined by measurement of immunoreactive PTHrP(1-86) in tissue explant supernatants, was increased 10-fold in RA versus OA synovial tissue. Synovial lining cells and fibroblast-like cells within the pannus expressed both PTHrP and the PTH/PTHrP receptor, findings that were confirmed by *in vitro* studies of cultured synoviocytes. TNF- $\alpha$  and IL-1 $\beta$  stimulated PTHrP expression in synoviocytes, while dexamethasone and interferon- $\gamma$ , agents with some therapeutic efficacy in the treatment of RA, inhibited PTHrP release. Treatment of synoviocytes with PTHrP(1-34) stimulated IL-6 secretion. These results suggest that proinflammatory cytokine-stimulated production of NH<sub>2</sub>-terminal PTHrP by synovial tissue directly invading cartilage and bone in RA may mediate joint destruction through direct effects on cartilage or bone, or, indirectly, via the induction of mediators of bone resorption in the tumor-like synovium. (*J. Clin. Invest.* 1998. 101:1362-1371.) **Key words:** tumor necrosis factor • interleukin 1 • interferon- $\gamma$  • osteoarthritis • synoviocytes

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### Introduction

Subsequent to the discovery of parathyroid hormone-related protein (PTHrP)<sup>1</sup> as the causative agent in humoral hypercalcemia of malignancy, studies have suggested that this peptide, when not overexpressed as a paraneoplastic manifestation of cancer, acts locally in an autocrine, paracrine, or intracrine fashion more akin to cytokines than to classic hormones, such as PTH (1-3). Previous work by Funk et al. has added a new dimension to this view of PTHrP as a cytokine-like peptide, demonstrating that PTHrP is a member of the cascade of proinflammatory cytokines that is induced in parenchymal organs in response to bacterial endotoxin (4-8). Just as TNF and IL-1 have been shown to mediate both beneficial and toxic effects during endotoxemia, NH<sub>2</sub>-terminal PTHrP shares with these agents the ability to induce the hepatic acute phase response, an important arm of the host defense against infection, as well as to contribute to endotoxin-induced mortality (7, 8). These studies have led us to hypothesize that PTHrP, like TNF and IL-1, may be an important mediator of inflammation in a variety of clinical settings, in addition to endotoxemia.

Although NH<sub>2</sub>-terminal PTHrP is known to mediate generalized bone resorption when present in high circulating levels in the setting of humoral hypercalcemia of malignancy, little is known about the possible role that a local, rather than systemic, induction of this cytokine-like peptide could play in mediating bone resorption associated with diseases other than neoplasia (1-3, 9). Given the well-described role of TNF and IL-1 in mediating bone resorption and the ability of these agents to stimulate PTHrP expression (4, 5, 9, 10), it is logical to postulate that local induction of PTHrP may also be occurring in clinical settings of cytokine-mediated bone resorption. However, although both PTHrP and the PTH/PTHrP receptor are produced constitutively in cartilage and in bone, little attention has been placed on the investigation of local changes in PTHrP expression at these sites in response to inflammation, or potential roles for PTHrP in cytokine-mediated bone disease (3, 9, 10). Indeed, a recent report demonstrating that production of NH<sub>2</sub>-terminal PTHrP by breast cancer cells, in the absence of any systemic effects, may contribute to the local destruction of bone in metastatic disease has provided the first real evidence suggesting that locally produced PTHrP may cause destruction of adjacent bone (11).

RA, a disease characterized by profound cytokine-mediated periarticular bone destruction, provides an excellent model for testing of the hypothesis that locally induced PTHrP mediates non-malignancy-related bone resorption in a manner analogous to other proinflammatory cytokines (12-14).

1. *Abbreviations used in this paper:* MMP, matrix metalloproteinase; OA, osteoarthritis; PTHrP, parathyroid hormone-related protein.

Because the aggressive overproduction of proinflammatory cytokines, such as TNF and IL-1, by the tumor-like synovium mediates the erosive destruction of cartilage and periarticular bone in RA, we therefore sought to determine whether expression of NH<sub>2</sub>-terminal PTHrP was also induced in the rheumatoid synovium, as compared with the less inflammatory osteoarthritic synovium. To determine whether PTHrP, in addition to its well-described catabolic effects on adjacent cartilage and bone (1–3, 15–18), could also act in a paracrine or autocrine fashion within the rheumatoid synovium, immunohistochemical studies were performed to identify sites of expression of PTHrP and the PTH/PTHrP receptor within the synovium. Given the importance of TNF and IL-1 in the pathogenesis of RA, regulation of PTHrP gene expression in cultured human synoviocytes in response to these proinflammatory cytokines, as well as to antiinflammatory agents, was also investigated. Lastly, to determine whether synovial PTHrP, in addition to direct effects on adjacent chondrocytes and osteoblasts (15–17), could also act in an autocrine fashion to induce mediators of joint destruction in synoviocytes, the effect of PTHrP(1-34) on synoviocyte production of IL-6, an important mediator of PTHrP-induced bone resorption, was also examined (18, 19).

## Methods

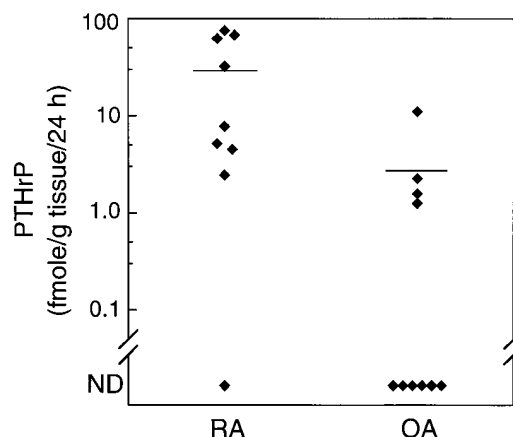
**Human synovial tissue specimens.** Synovial tissue samples, collected under sterile conditions and kept at 4°C for < 1 h before processing, were obtained from patients with RA ( $n = 9$ ) or osteoarthritis (OA) ( $n = 10$ ) at the time of joint replacement surgery under a protocol approved by the University of Arizona Human Subjects Committee. Samples, with the exception of one RA sample obtained from a shoulder joint, were obtained from the knee or hip. All of the tissue samples were processed and cultured to generate tissue explant supernatants. Identification of synovial tissue was confirmed by visualization of characteristic fronds in all tissue sections isolated by phase-contrast microscopy. In addition, sections of several of the RA ( $n = 5$ ) and OA ( $n = 3$ ) tissue samples were fixed in 10% formalin for immunohistochemical studies. Primary cultures of synoviocytes were also generated from synovial tissue samples studied ( $n = 2$ ), as well as from synovial tissue isolated from additional patients, including one nonarthritic patient with joint trauma undergoing arthroscopic surgery ( $n = 5$ ).

**Synoviocyte isolation and culture.** Primary cultures of human synoviocytes were established using standard methods as previously described (20). Cells were passaged when confluent using DME/F12 plus penicillin (100 U/ml), streptomycin (100 U/ml), and glutamine (2 mM) (Fisher, Tustin, CA) (medium A), supplemented with 10% defined FCS (Hyclone Laboratories, Logan, UT). Cell lines, which were isolated from four RA (designated RA1-4), two OA (OA1-2), and one nonarthritic (NL1) synovial samples, were used between the second and sixth passage and were fibroblast-like in appearance. For experiments in which cytokine-induced changes in synoviocyte morphology were evaluated, cells were examined by phase-contrast microscopy and the effect of treatments on transformation to a stellate morphology was determined by counting the number of stellate cells per field using a  $\times 20$  objective. Stellate synoviocytes, which have been well-described by several other laboratories (21–25), were easily identified by their characteristic long, thin projections extending from a rounded central body, as contrasted with the broadly spread cytoplasm of untreated, confluent, fibroblast-like synoviocytes.

**Immunoradiometric assay of PTHrP.** Immunoreactive PTHrP was assayed in tissue or cell supernatants using a two-site immunoradiometric assay that uses antibodies directed against synthetic human PTHrP(1-40) and PTHrP(60-72) (Nichols Institute, San Juan Capis-

trano, CA). PTHrP(1-86) standards were diluted in the same media used for samples. For determination of PTHrP secretion from tissue explants, synovial tissue specimens were processed within 1 h of isolation using apyrogenic instruments and supplies. Minced synovial tissue was weighed and distributed to the wells of 24-well Falcon tissue culture plates for 24 h of incubation in 300  $\mu$ l of medium A supplemented with 2% FCS/well ( $n = 3 \pm 1$  wells/tissue sample). Average wet tissue weight/well ( $45 \pm 0.2$  vs.  $55 \pm 2$  mg for RA and OA tissue, respectively) was slightly higher for OA tissue, thus enhancing our ability to detect lower levels of PTHrP secretion in these tissues. Tissue supernatant PTHrP levels < 0.3 pM were below the sensitivity of the assay. For calculation of mean PTHrP release in RA as compared with OA tissues, samples with undetectable PTHrP were assigned a tissue supernatant PTHrP value of 0.3 pM for calculation of femtomoles of PTHrP released per gram of tissue over 24 h. For determination of PTHrP production by synoviocytes, cells were plated at  $10^5$  cells/well in 24-well tissue culture plates in medium A supplemented with 10% FCS on the day before the start of the experiment. Cells were then incubated with 300  $\mu$ l of medium A supplemented with 2% FCS with or without the indicated doses of cytokines or dexamethasone. Because the dexamethasone stock was dissolved in ethanol, all treatment conditions for experiments involving dexamethasone had a final ethanol content of 0.01%. Human recombinant TNF- $\alpha$ , IFN- $\gamma$ , and IL-6 (Intergen, Purchase, NY), human recombinant IL-1 $\beta$  (Becton Dickinson, Bedford, MA), and dexamethasone (Sigma Chemical Co., St. Louis, MO) were all obtained from commercial sources. To prevent proteolysis of PTHrP (26), protease inhibitors were added to tissue and cell supernatants at the time of harvest (5.0  $\mu$ g/ml aprotinin and 2.5  $\mu$ g/ml leupeptin; Boehringer Mannheim, Indianapolis, IN) and the samples were immediately frozen and stored at  $-20^\circ\text{C}$  before assay.

**Immunohistochemistry.** Synovial tissue specimens were fixed with 10% formalin, embedded in paraffin, cut into 5- $\mu$ m sections, and deparaffinized for immunohistochemical staining. Synoviocytes, grown on apyrogenic glass coverslips, were treated with methanol for 15 min at 4°C before staining. Using previously described methods (8), tissue or cell samples were processed for detection of PTHrP using 1.5–2.5  $\mu$ g/ml affinity-purified rabbit polyclonal antibody generated against human PTHrP(34-53) (Oncogene Science, Cambridge, MA), or for



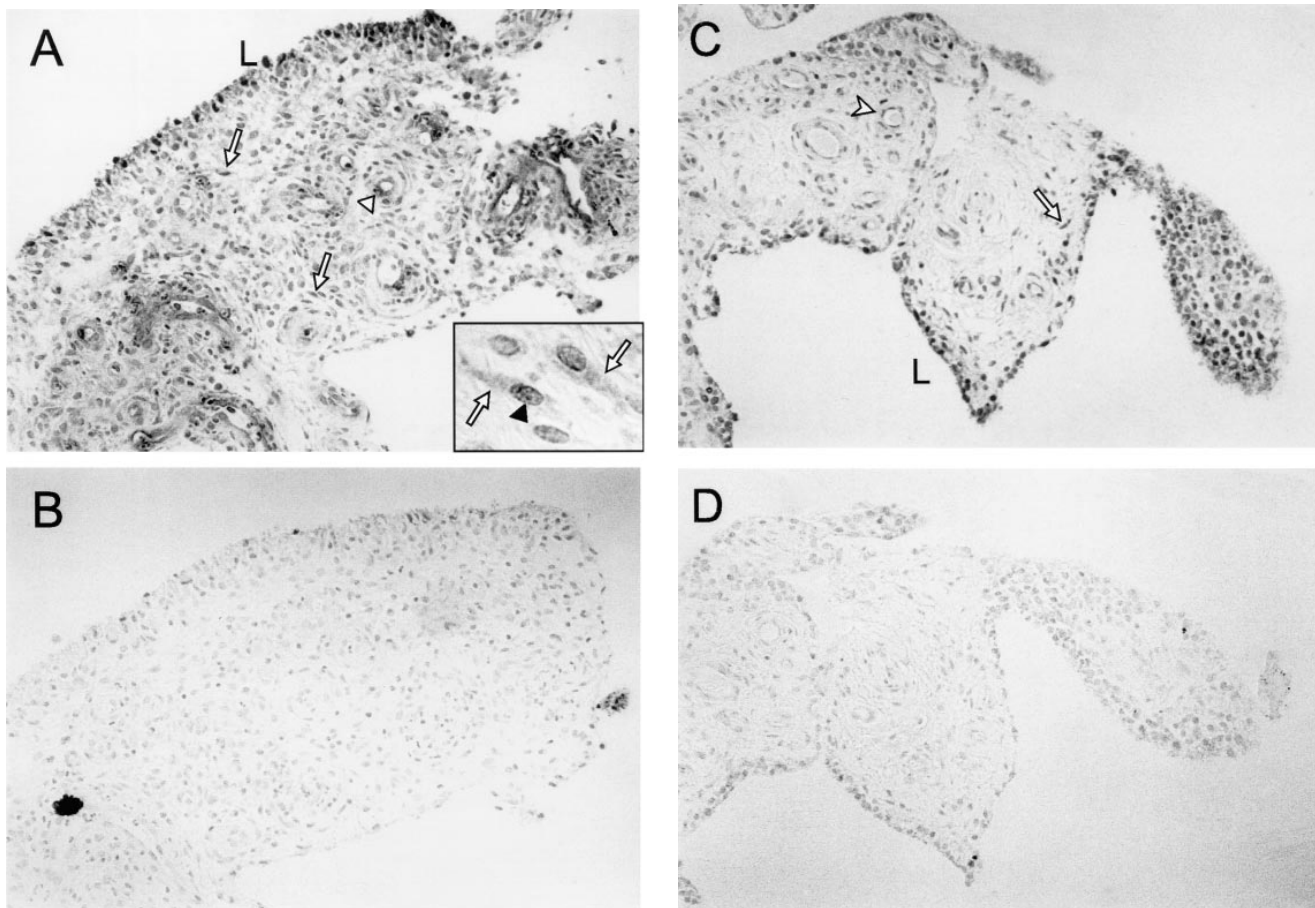
**Figure 1.** PTHrP production by RA and OA synovial tissue. After 24 h of incubation with fresh synovial tissue explants, tissue supernatant PTHrP(1-86) levels were measured by immunoradiometric assay as described in Methods. Individual values are reported on a log scale as mean femtomoles of PTHrP released per gram of tissue per 24 h for each synovial tissue sample ( $n = 3$ –4 explants per synovial tissue sample). ND, None detected, with lines indicating mean PTHrP detected in RA vs. OA samples ( $P < 0.02$ ).

detection of the PTH/PTHrP receptor using 3–5  $\mu\text{g}/\text{ml}$  affinity-purified rabbit polyclonal antibody generated against an extracellular portion of the rat receptor (amino acids 90–106) which is 70% homologous with the human protein (antibody and antigen kindly provided by Dr. Robert Nissenson, University of California, San Francisco, CA). Specificity of staining for PTHrP or the receptor was confirmed by the absence of staining that resulted when serial sections were incubated with primary antibody that had been preincubated overnight at 4°C with a 40–50-fold excess by weight of PTHrP(34–53) peptide (Oncogene Science) or a 20-fold excess of PTH/PTHrP receptor(90–106) peptide, respectively, or with rabbit IgG. In tissue sections, nuclei were stained with methyl green.

**Northern blot analysis.** Cells were grown to confluency in 150-mm Corning tissue culture dishes or T75 flasks. For experiments, cells were incubated for the indicated amount of time with or without cytokines in medium A supplemented with 2% FCS. At the time of harvest, cells were washed with ice-cold PBS and total RNA was then isolated using the Ultraspec RNA isolation system (Biotex, Houston, TX). Additionally, in some experiments, polyadenylated RNA was also isolated from synoviocytes for detection of PTH/PTHrP receptor mRNA. For isolation of polyadenylated RNA, cells were washed with ice-cold PBS, dissolved in a solution of 10 mM Tris, pH 7.5, 1 mM EDTA, and 1% SDS (solution A) containing 0.5 M NaCl and 200  $\mu\text{g}/\text{ml}$  proteinase K, and then incubated with type 77F

oligo(dT) cellulose (Pharmacia Biotech, Piscataway, NJ). After washing with high and low salt buffers (solution A containing 0.5 M NaCl or 0.1 M NaCl), polyadenylated RNA was eluted from the oligo(dT) cellulose. Using previously described methods (4), total or polyadenylated RNA was fractionated in 1% agarose gels containing 2.2 M formaldehyde, transferred to nylon membranes electrophoretically, and hybridized to random prime-labeled [ $^{32}\text{P}$ ]cDNA probes. Blots were probed using EcoRI-EcoRI human PTHrP 10B5 clone cDNA (27), BamHI-XhoI human PTH/PTHrP receptor (HKrk) cDNA (28), and actin cDNA kindly provided by Dr. Mark Thiede (Pfizer, Groton, CT), Dr. Harald Jüppner (Massachusetts General Hospital, Harvard Medical School, Boston, MA), and Dr. Peter Gunning (Stanford University, Stanford, CA), respectively. Blots were exposed to film at  $-70^\circ\text{C}$  using intensifying screens, and autoradiographic intensity was quantitated using an imaging densitometer (model GS-700; Bio-Rad, Hercules, CA).

**IL-6 immunoassay.** For detection of IL-6 release, synoviocytes were plated as in the PTHrP release experiments. Cells were then incubated for 24 h in 300  $\mu\text{l}$  of medium A supplemented with 2% FCS with or without the indicated doses of PTHrP(1–34), a peptide that binds to and activates the PTH/PTHrP receptor (Bachem, Torrance, CA). PTHrP peptide stocks contained no detectable endotoxin ( $< 5$  pg endotoxin/mg peptide), as determined by Limulus assay using previously described methods (5). Cell supernatants were frozen imme-



**Figure 2.** Immunohistochemical staining for PTHrP in RA and OA synovial tissue. Paraffin-embedded sections of RA (A) or OA (C) synovial tissue were stained with antibody directed against PTHrP(34–53). Specificity of staining was confirmed by comparison with serial sections of RA (B) or OA (D) synovial tissue treated with PTHrP(34–53) antibody that had been preincubated with an excess of antigen. Nuclei were stained with methyl green. Synovial lining cells (L) and fibroblast-like cells within the tissue (arrows) stained for PTHrP, with specific staining appearing to occur in the cytoplasm as well as the nucleus of the cells (A, inset, with nuclear stain indicated by a dark arrowhead). Specific PTHrP staining was also seen in the synovial vasculature (white arrowhead).

diately after harvest and stored at  $-20^{\circ}\text{C}$  before assay. Supernatant levels of IL-6 were assayed using a commercially available human IL-6 ELISA (Genzyme, Cambridge, MA). IL-6 was not detected in unconditioned media (assay sensitivity 18 pg/ml).

**Statistical analysis.** Values are presented as mean  $\pm$  SEM, with statistical significance determined using InStat 2.01 statistical software (Graphpad Software, San Diego, CA).

## Results

**Production of NH<sub>2</sub>-terminal PTHrP by human synovial tissue ex vivo.** Synovial tissue samples, obtained from the joints of patients with RA ( $n = 9$ ) or OA ( $n = 10$ ), were cultured ex vivo and NH<sub>2</sub>-terminal PTHrP levels in tissue supernatants were measured after 24 h of incubation. While only 40% of the OA tissues produced measurable PTHrP, PTHrP release was detected in all but one of the RA synovial tissues (Fig. 1). RA synovium produced 10-fold more PTHrP than did OA synovium ( $29.04 \pm 10.54$  vs.  $2.71 \pm 0.94$  fmol/g tissue/24 h,  $P < 0.02$ ) (Fig. 1).

**Immunohistochemical localization of PTHrP in human synovial tissue.** Specific staining for PTHrP was seen in all RA ( $n = 5$ ) and OA ( $n = 3$ ) tissues studied. In both RA (Fig. 2 A) and OA synovial tissue (Fig. 2 C), specific PTHrP staining was most intense in the synovial lining, which is composed of type A (macrophage-like) and type B (fibroblast-like) synoviocytes (13). In RA synovium, numerous fibroblast-like cells within the synovium also stained specifically for PTHrP (Fig. 2 A, inset, arrows), while only occasional fibroblast-like cells in the hypocellular OA synovium were PTHrP positive. Similarly, the synovial vasculature, including endothelial cells, was also a site of PTHrP staining in all tissues, although staining was more intense in RA synovial vessels (Fig. 2, A and C, white arrowheads). Specificity of staining for PTHrP in RA and OA tissue was verified by the absence of staining found on serial sections treated with PTHrP(34-53) antibody that had been preincubated with an excess of PTHrP(34-53) antigen (Fig. 2, B and D, respectively). In contrast, although occasional mononuclear cells appeared to stain positively for PTHrP, promi-

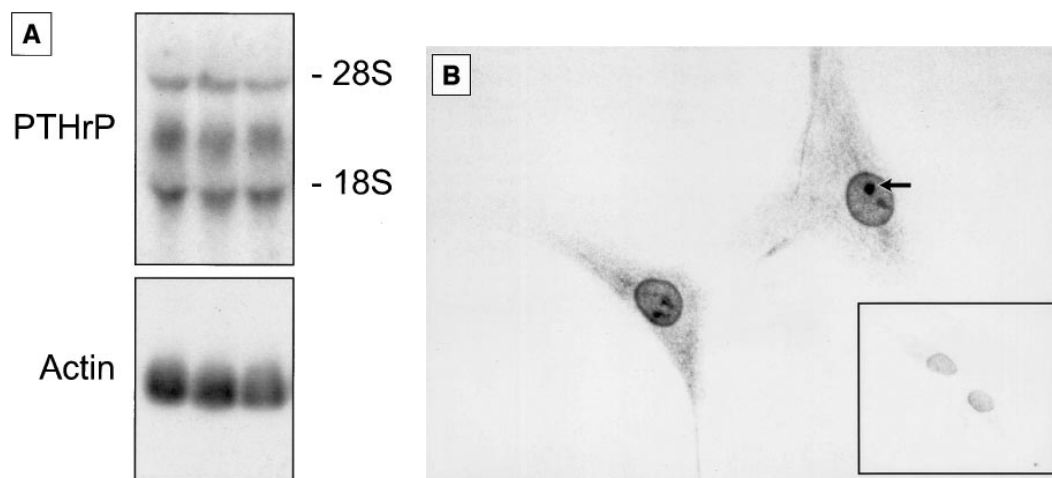
**Table I. Effect of TNF- $\alpha$ , IL-1 $\beta$ , or a Combination of TNF- $\alpha$  and IL-1 $\beta$  on PTHrP Secretion from Synoviocytes Isolated from RA, OA, or Nonarthritic (NL) Joints**

| Cell type | PTHrP (pM)      |                            |                             |                              |
|-----------|-----------------|----------------------------|-----------------------------|------------------------------|
|           | Control         | TNF- $\alpha$              | IL-1 $\beta$                | TNF- $\alpha$ + IL-1 $\beta$ |
| RA        | $3.02 \pm 0.14$ | $5.92 \pm 0.58^*$          | $9.97 \pm 0.16^{\ddagger}$  | $7.02 \pm 0.34^{\ddagger}$   |
| OA        | $2.65 \pm 0.27$ | $4.66 \pm 0.28^{\S}$       | $12.78 \pm 0.56^{\ddagger}$ | $12.81 \pm 0.52^{\ddagger}$  |
| NL        | $2.63 \pm 0.13$ | $6.65 \pm 0.22^{\ddagger}$ | $8.08 \pm 1.02^{\ddagger}$  | —                            |

Cells were treated with 10 ng/ml TNF- $\alpha$ , 10 ng/ml IL-1 $\beta$ , a combination of 10 ng/ml TNF- $\alpha$  and IL-1 $\beta$ , or media alone. Supernatant PTHrP levels, reported as mean  $\pm$  SEM, were determined by IRMA as described in Methods ( $n = 3-4$ /condition).  $^{\S}P < 0.05$  vs. control;  $^*P < 0.01$  vs. control;  $^{\ddagger}P < 0.001$  vs. control.

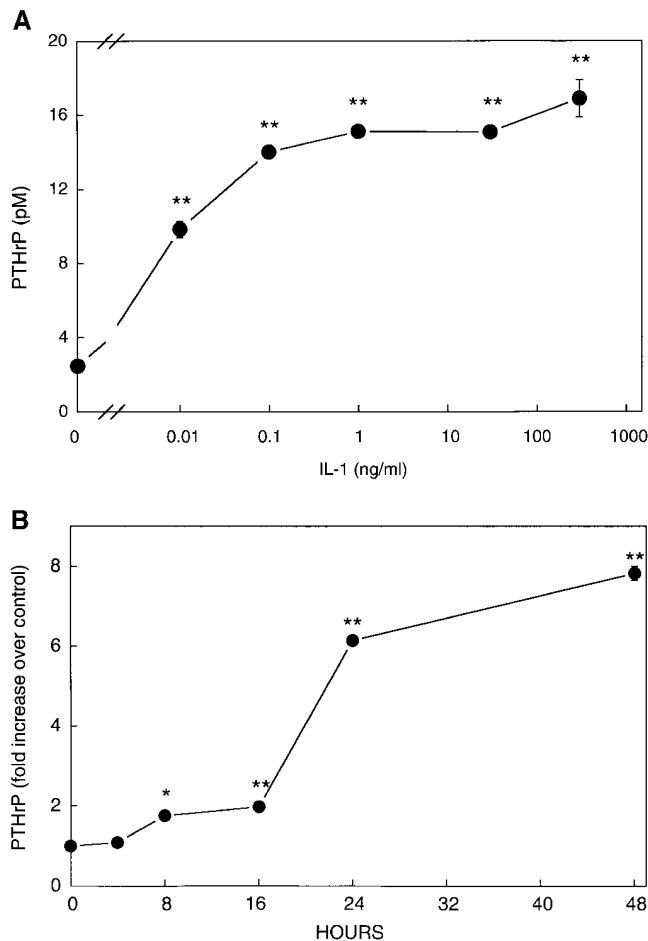
nent mononuclear cell infiltrates in RA synovium were PTHrP negative (data not shown).

**Expression of PTHrP by human synoviocytes.** Because synoviocytes, which stained specifically for PTHrP in human synovium, are believed to be an important source of proinflammatory cytokines and mediators of bone resorption in RA (12-14), additional in vitro experiments were conducted to verify PTHrP production by these cells. PTHrP gene expression was confirmed by detection of PTHrP mRNA in cultured RA synoviocytes (Fig. 3 A), as well as OA synoviocytes (data not shown). Immunohistochemical studies localized PTHrP protein in the cytoplasm as well as discreet areas of the nucleus of these cells (Fig. 3 B), with specificity of staining being demonstrated by the absence of staining seen when PTHrP antibody was preincubated with an excess of antigen (Fig. 3 B, inset). Examination of higher magnification views of PTHrP-positive fibroblast-like cells within the RA synovium suggests that a similar staining pattern may be seen in vivo (Fig. 2 A, inset, dark arrowhead). The same immunohistochemical staining pattern was seen in multiple RA and OA cell lines. In addi-



**Figure 3. PTHrP expression in rheumatoid synoviocytes.** (A) Northern analysis of PTHrP in cultured rheumatoid synoviocytes. Total RNA was isolated from untreated rheumatoid synoviocytes (RA2; passage 4) and assayed by Northern analysis (24  $\mu\text{g}$ /lane) for expression of PTHrP and actin as described in Methods. Three major PTHrP transcripts, the result of alternative splicing, are seen (1). (B) Immunohistochemical staining of PTHrP in

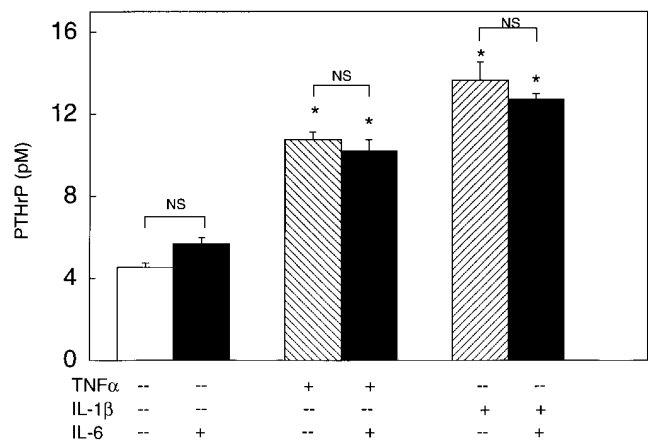
rheumatoid synoviocytes. Cultured rheumatoid synoviocytes (RA4; passage 6) were stained for PTHrP as described in Methods. Specific staining for PTHrP was seen in the cytoplasm as well as discreet areas of the nucleus (see arrow). Specificity of staining was determined by comparison with cells stained with PTHrP(34-53) antibody that had been preincubated with an excess of antigen (see inset).



**Figure 4.** IL-1 induction of PTHrP release from rheumatoid synoviocytes. (A) IL-1 dose-response. Rheumatoid synoviocytes (RA4; passage 4) were treated for 24 h with the indicated doses of IL-1 $\beta$ . Cell supernatant PTHrP(1-86) levels, measured by IRMA as described in Methods, are reported as mean  $\pm$  SEM ( $n = 3$ –5/condition). (B) IL-1 time course. Rheumatoid synoviocytes (RA4; passage 4) were treated for the indicated times with 30 ng/ml IL-1 $\beta$  or with media alone (control). Cell supernatant PTHrP(1-86) levels are reported as fold-change over control cells at each time point (mean  $\pm$  SEM with  $n = 3$ –5/condition). Differences in A and B are statistically significant by ANOVA. For data points where standard error bars are not visible, the bars fall within the dot. \* $P < 0.01$  vs. control. \*\* $P < 0.001$  vs. control.

tion, production and release of NH<sub>2</sub>-terminal PTHrP protein was confirmed by detection of immunoreactive PTHrP in cell supernatants obtained from cultured RA, OA, and nonarthritic synoviocytes (Table I, control). Constitutive PTHrP release, which remained fairly constant over consecutive passages, was detected in all synoviocyte cell lines examined ( $n = 7$ ).

**Effect of proinflammatory cytokines on NH<sub>2</sub>-terminal PTHrP release by synoviocytes.** TNF- $\alpha$  and IL-1 $\beta$  significantly induced the release of NH<sub>2</sub>-terminal PTHrP by synoviocytes isolated from RA, OA, or nonarthritic joints (Table I). Coadministration of TNF and IL-1 did not augment the effects of IL-1 on PTHrP release (Table I). Because IL-1 consistently appeared to be a more potent inducer of PTHrP than did TNF, the effect of IL-1 on PTHrP release was further evaluated. IL-1

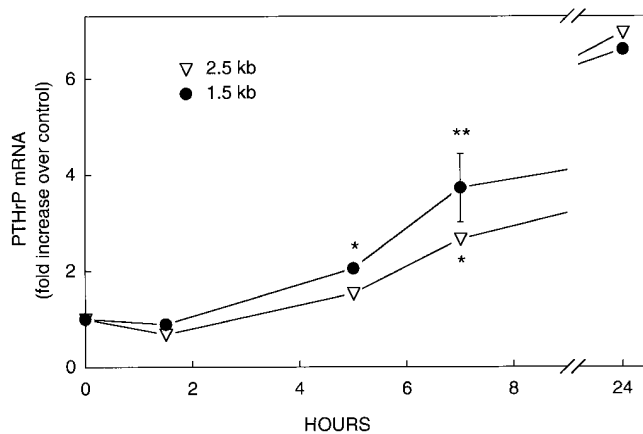


**Figure 5.** Effect of IL-6 on PTHrP release from rheumatoid synoviocytes. Rheumatoid synoviocytes (RA1; passage 4) were treated for 24 h with 20 ng/ml TNF- $\alpha$ , 20 ng/ml IL-1 $\beta$ , 200 ng/ml IL-6, a combination of TNF- $\alpha$  (20 ng/ml) and IL-6 (200 ng/ml), a combination of IL-1 $\beta$  (20 ng/ml) and IL-6 (200 ng/ml), or media alone. PTHrP(1-86) levels in supernatants, measured as described in Methods, are reported as mean  $\pm$  SEM ( $n = 3$ /condition). Differences are statistically significant by ANOVA. \* $P < 0.001$ , vs. untreated control cells. NS, Not significant.

stimulated PTHrP release in a dose-dependent fashion, with maximal induction occurring with doses of IL-1 as low as 100 pg/ml (Fig. 4 A). PTHrP levels in cell supernatants were increased by 8 h after IL-1 treatment, and continued to increase for up to 48 h (Fig. 4 B). IL-6 is also an important member of the cytokine cascade produced in the rheumatoid synovium (12–14, 19, 29). However, in contrast to the marked effects of TNF and IL-1, treatment of cells with up to 200 ng/ml IL-6, in the absence or presence of TNF or IL-1, did not affect PTHrP release (Fig. 5).

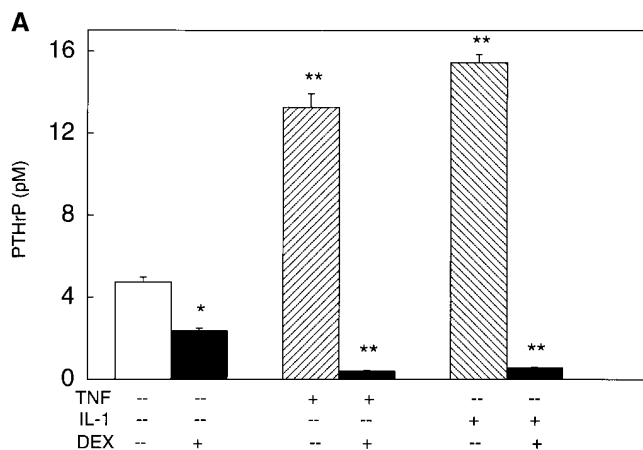
**Effect of IL-1 on PTHrP gene expression in synoviocytes.** To determine if IL-1 stimulation of PTHrP release was associated with an increase in PTHrP gene expression, the effect of IL-1 on PTHrP mRNA levels in synoviocytes was determined by Northern analysis. IL-1 (10 ng/ml) markedly increased PTHrP mRNA levels in RA synoviocytes (Fig. 6). PTHrP mRNA levels were induced as early as 5 h after initiation of treatment with IL-1, thus preceding the observed increase in PTHrP protein release (Fig. 4 B), and persisted until the latest time point studied (24 h).

**Effect of dexamethasone on NH<sub>2</sub>-terminal PTHrP release by synoviocytes.** The effect of corticosteroids, potent inhibitors of proinflammatory cytokines, on constitutive and stimulated NH<sub>2</sub>-terminal PTHrP release by cultured synoviocytes was determined (30–33). As can be seen in Fig. 7 A, treatment of RA synoviocytes with dexamethasone inhibited constitutive PTHrP release. Similarly, dexamethasone also inhibited TNF- and IL-1-stimulated PTHrP release (Fig. 7 A). Induction of PTHrP release (Fig. 7 A) was closely associated with a cytokine-mediated transformation of cells from a fibroblast-like to a stellate morphology (Fig. 7 B) that has been reported to be associated with an increased release of mediators of bone resorption (21–25). This change in synoviocyte morphology occurred in all cell lines examined ( $n = 6$ ) in both early (passage 2) and late (passage 6) passage cells, resulting in 80–100% stellate cells after 24 h of treatment with IL-1 (data not shown).

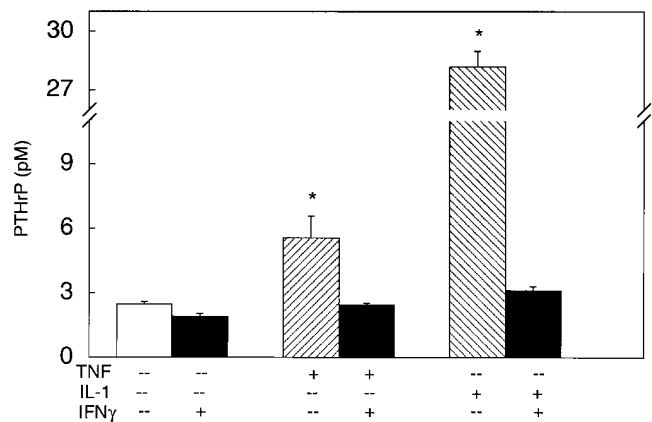


**Figure 6.** Time course of IL-1 induction of PTHrP mRNA levels in rheumatoid synoviocytes. Rheumatoid synoviocytes (RA4; passage 4) were treated with 10 ng/ml IL-1 $\beta$  or media alone for the indicated times. Total RNA was isolated and assayed by Northern analysis (22  $\mu$ g/lane) for expression of PTHrP and actin as described in Methods. PTHrP mRNA levels (mean  $\pm$  SEM), expressed as arbitrary densitometry units of PTHrP/actin in treated cells divided by PTHrP/actin in untreated cells at each time point, are shown for the two smallest and most prominent PTHrP mRNA transcripts ( $\sim$  2.5 kb, triangles, and 1.5 kb, circles, as determined by comparison with location of 28S and 18S rRNA). All time points had  $n = 2$ –3/condition, except for the 24-h time point ( $n = 1$ /condition). With the exception of the 24-h time point, where standard error bars are not visible, the bars fall within the dot. Statistical significance for time points with  $n \geq 2$ /condition was calculated by ANOVA. \* $P < 0.01$  vs. untreated. \*\* $P < 0.001$  vs. untreated.

Dexamethasone inhibited both PTHrP release as well as the transformation of synoviocytes from a fibroblast-like to a stellate morphology (Fig. 7, A and B). In contrast to the effects of TNF and IL-1, treatment of synoviocytes with PTHrP(1-34) in doses as high as  $10^{-6}$  M had no effect on cell shape (data not



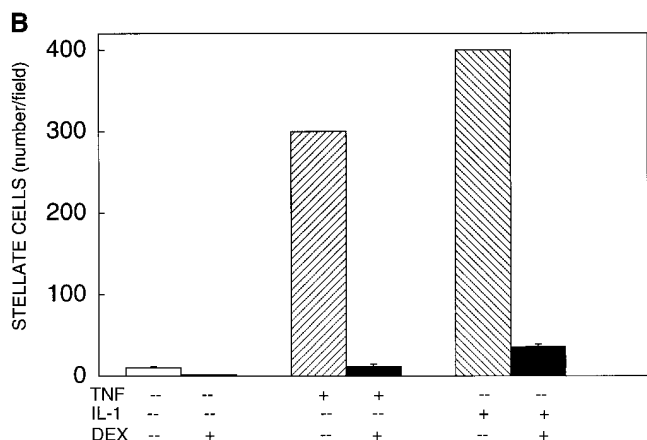
**Figure 7.** Effect of dexamethasone on PTHrP release (A) and synoviocyte morphology (B). Rheumatoid synoviocytes (RA2; passage 3) were treated with 30 ng/ml TNF- $\alpha$ , 30 ng/ml IL-1 $\beta$ ,  $10^{-7}$  M dexamethasone, a combination of TNF- $\alpha$  (30 ng/ml) and dexamethasone ( $10^{-7}$  M), a combination of IL-1 $\beta$  (30 ng/ml) and dexamethasone ( $10^{-7}$  M), or media alone. (A) After 24 h, supernatants were harvested for measurement of PTHrP(1-86), reported as mean  $\pm$  SEM ( $n = 4$ /condition), as described in Methods. Differences are statistically significant by ANOVA. \* $P < 0.01$  vs. untreated control cells. \*\* $P < 0.001$  vs. untreated control cells. (B) Immediately before harvesting supernatants, cell morphology was evaluated by phase-contrast microscopy using a  $\times 20$  objective. The number of stellate cells per field, reported as mean  $\pm$  SEM, was counted and averaged over two to four fields for each treatment condition.

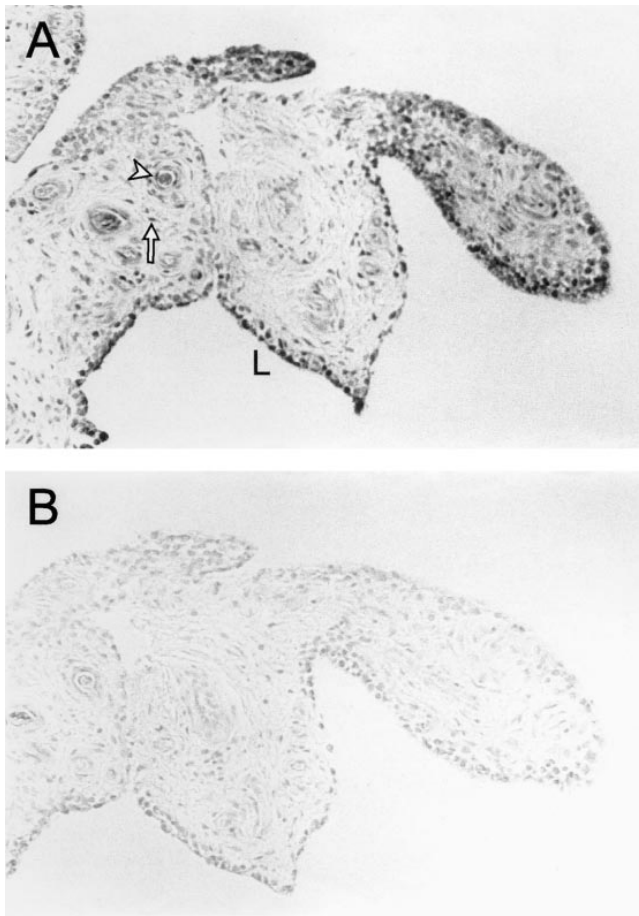


**Figure 8.** Effect of IFN- $\gamma$  on PTHrP release by synoviocytes. Synoviocytes isolated from an osteoarthritic joint (OA1; passage 3) were treated for 24 h with 30 ng/ml TNF- $\alpha$ , 30 ng/ml IL-1 $\beta$ , 30 ng/ml IFN- $\gamma$ , a combination of TNF- $\alpha$  (30 ng/ml) and IFN- $\gamma$  (30 ng/ml), a combination of IL-1 $\beta$  (30 ng/ml) and IFN- $\gamma$  (30 ng/ml), or media alone. PTHrP(1-86) concentration in supernatants, measured as described in Methods, is reported as mean  $\pm$  SEM ( $n = 4$ /condition). Differences are statistically significant by ANOVA. \* $P < 0.001$  vs. untreated control cells.

shown), thus suggesting that PTHrP does not mediate the effect of these cytokines on cell morphology.

**Effect of IFN- $\gamma$  on NH $_2$ -terminal PTHrP release by synoviocytes.** While IFN- $\gamma$  is classically thought of as a proinflammatory cytokine that acts synergistically with TNF and IL-1, many of the proinflammatory effects of TNF and IL-1 on synoviocytes are paradoxically inhibited by this Th $_1$  cell product (31, 32, 34). Therefore, the effect of this agent on TNF- and IL-1-stimulated NH $_2$ -terminal PTHrP release from synoviocytes was investigated. As can be seen in Fig. 8, IFN- $\gamma$  alone had no effect on constitutive PTHrP release from cultured





**Figure 9.** Immunohistochemical staining for PTH/PTHrP receptor in synovial tissue. Paraffin-embedded section of OA synovial tissue stained with antibody directed against the PTH/PTHrP receptor (A). Specificity of staining was confirmed by comparison with serial sections treated with receptor antibody that had been preincubated with an excess of antigen (B). Nuclei were stained with methyl green. Synovial lining cells (L), fibroblast-like cells within the tissue (arrow), and the synovial vasculature (arrowhead) stained specifically for the PTH/PTHrP receptor. Distribution of staining for PTH/PTHrP receptor can be compared with PTHrP localization on a serial section of the same tissue (Fig. 2 C).

synoviocytes. However, both TNF and IL-1 induction of PTHrP release was completely inhibited by concurrent treatment with IFN- $\gamma$ .

**Immunohistochemical localization of PTH/PTHrP receptor in human synovial tissue.** To determine whether NH<sub>2</sub>-terminal PTHrP, in addition to its well-described catabolic effects on bone and cartilage (1–3, 15–18), could also act in an auto-crine or paracrine fashion within the human synovium, immunohistochemical studies were undertaken to determine whether the PTH/PTHrP receptor was expressed in the human synovium. Specific staining for the PTH/PTHrP receptor was seen in all RA ( $n = 5$ ) and OA ( $n = 3$ ) tissues studied. In both RA (not shown) and OA tissues (Fig. 9 A), the distribution of specific staining for the PTH/PTHrP receptor was the same as that seen for PTHrP itself (Fig. 2 C); PTH/PTHrP receptor was present in synovial lining cells, fibroblast-like cells within

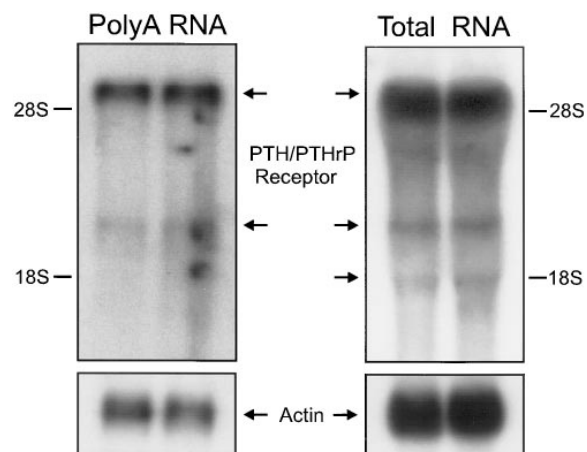
the synovium, and the synovial vasculature. Specificity of staining was verified by the absence of staining found on serial sections treated with PTH/PTHrP receptor antibody that had been preincubated with an excess of antigen (Fig. 9 B).

**PTH/PTHrP receptor expression in human synoviocytes.** Expression of PTH/PTHrP receptor by human synoviocytes was confirmed by Northern analysis of cultured synoviocytes. The most abundant PTH/PTHrP receptor RNA transcript detected in Northern blots of total and polyadenylated synoviocyte RNA was  $\sim 5$  kb (Fig. 10), a size similar to that reported for the PTH/PTHrP receptor in human keratinocytes, but much larger than the major 2.3-kb transcript encoding the human PTH/PTHrP receptor in kidney and bone, the classic sites of PTH action (35). However, additional smaller, but much less abundant, transcripts were also detected (Fig. 10).

**Effect of PTHrP(1-34) on synoviocyte IL-6 release.** PTH/PTHrP activation of osteoclastic bone resorption is thought to occur indirectly via PTH/PTHrP receptor-mediated stimulation of osteoblastic IL-6 production (18). To determine whether synoviocytes could also be a source of PTHrP-stimulated IL-6 release in RA, the effect of PTHrP(1-34) on IL-6 production by cultured synoviocytes was measured. Treatment of synoviocytes with  $10^{-9}$  M PTHrP(1-34), a peptide that binds to and activates the PTH/PTHrP receptor, significantly increased IL-6 release ( $320.2 \pm 28.8$  vs.  $531.3 \pm 65.7$  pg/ml,  $P < 0.01$ ). Similar results were obtained in three separate experiments, using two different RA cell lines (data not shown).

## Discussion

RA provides an extreme example of localized proinflammatory cytokine-mediated destruction of cartilage and bone for investigation of a potential role for localized PTHrP expression in cytokine-mediated bone resorption. In the studies presented here, rheumatoid synovial tissue, the local source of joint-destroying cytokines in RA, produced markedly in-



**Figure 10.** PTH/PTHrP receptor expression in rheumatoid synoviocytes. Polyadenylated RNA or total RNA, isolated from two untreated rheumatoid synoviocyte cell lines (RA2 passage 3, and RA3 passage 6, respectively), was assayed by Northern analysis (3 or 6  $\mu$ g polyadenylated RNA and 24  $\mu$ g total RNA per lane) for expression of PTH/PTHrP receptor and actin as described in Methods. Messenger RNA transcripts are indicated by arrows.



creased amounts of NH<sub>2</sub>-terminal PTHrP, a resorptive agent that binds to and activates the PTH/PTHrP receptor, as compared with the less inflammatory OA synovium. Synovial NH<sub>2</sub>-terminal PTHrP production thus mirrors the profiles of synovial expression of other proinflammatory cytokines, such as TNF, IL-1, and IL-6, which, although present in both diseases, appear to be produced at much higher levels in RA as compared with the less destructive and less inflammatory osteoarthritic disease process (13). The potential physiologic significance of increased NH<sub>2</sub>-terminal PTHrP production per weight of tissue in RA is increased further when one considers the fact that significantly more synovial tissue is present in patients with active RA, as compared with OA, due to the tumor-like proliferation of synovial tissue in this disease (12).

This finding of increased NH<sub>2</sub>-terminal PTHrP release by RA versus OA synovial tissue can be contrasted with the results of two recent studies, appearing in the literature during the preparation of this manuscript, which reported the detection of measurable PTHrP in human synovial fluid, as well as the immunohistochemical localization of PTHrP in synovial tissue (36, 37). These studies, while detecting NH<sub>2</sub>-terminal PTHrP peptides in human synovial fluid, did not find an increase in RA versus OA and/or normal samples. The apparent disparity between NH<sub>2</sub>-terminal PTHrP production by rheumatoid synovial tissue versus synovial fluid PTHrP levels could be explained by several factors. Firstly, cytokines present in synovial fluid are not necessarily derived from synovial tissue, but rather are produced from a variety of sources and therefore do not accurately reflect levels of expression by the synovium, the tissue that is directly invading adjacent cartilage and bone (12–14, 20, 29). Additionally, because NH<sub>2</sub>-terminal PTHrP peptides containing both PTHrP(1–34) and midregion epitopes, as assayed in these studies, are extremely susceptible to degradation (26), the finding of low levels of these peptides in endogenous protease-containing synovial fluid isolated from RA, OA, and normal joints may simply reflect enhanced proteolysis rather than decreased production (13, 29). Indeed, it should be noted that while NH<sub>2</sub>-terminal PTHrP peptides were not found to be increased in RA synovial fluid, both studies did report an increase in COOH-terminal peptides of this posttranslationally processed protein (3), an increase that correlated with other markers of inflammation (36, 37).

Immunohistochemical localization of PTHrP in synoviocytes and the synovial vasculature confirms previous reports localizing PTHrP in the human synovium and demonstrates that PTHrP is produced at the same synovial sites as other important proinflammatory cytokines, such as TNF, IL-1, and IL-6 (12, 13, 20, 29, 36–39). Moreover, the demonstration in sequential synovial tissue sections of an identical pattern of immunohistochemical localization of PTHrP and the PTH/PTHrP receptor in synoviocytes, cells which are believed to be a major source of mediators of joint destruction in RA and are at the leading edge of invasive erosion into adjacent cartilage and bone (12–14), provides compelling evidence to support the hypothesis that the increased NH<sub>2</sub>-terminal PTHrP produced in RA could be acting locally in an autocrine or paracrine fashion within the synovium, as well as acting directly on adjacent cartilage and bone.

Expression of NH<sub>2</sub>-terminal PTHrP by human synoviocytes was confirmed by *in vitro* studies of cultured cells. As has been reported for other proinflammatory cytokines (19, 40), unstimulated synoviocytes isolated from RA, OA, and nonar-

thritic joints all produced similar amounts of PTHrP. It cannot be established whether this unstimulated release is an artifact of culture or is representative of *in vivo* production rates. What is made very clear by these *in vitro* studies, however, is the marked ability of TNF and IL-1, two cytokines that are induced locally in RA, and, to a lesser extent, in OA, to stimulate PTHrP release by synoviocytes isolated from RA, OA, and nonarthritic joints. Moreover, IL-1-stimulated PTHrP release was preceded by an increase in synoviocyte PTHrP mRNA levels, consistent with IL-1 activation of PTHrP gene expression. In addition, immunohistochemical studies of cultured cells located PTHrP in the nucleus as well as the cytoplasm of synoviocytes, a staining pattern that was also discernible to a lesser extent in the less antigenic, formalin-fixed paraffin synovial tissue sections. This finding is of particular interest in light of a previous report that nucleolar PTHrP may act in an intracrine fashion to regulate apoptosis, a process that is thought to occur with increased frequency in the rheumatoid synovium (41, 42).

Previous studies have reported that the cytokine-stimulated release of resorptive agents, such as matrix metalloproteinases (MMPs) and PGE<sub>2</sub>, by synoviocytes occurs in association with a change from a fibroblast-like to a stellate morphology (22–24). Indeed, induction of a change in cytoskeletal architecture by treatment with cytochalasin B is a sufficient stimulus for MMP production (25). Cytokine induction of PTHrP release also occurred in parallel with a transformation of synoviocytes from a fibroblast-like to a stellate shape. Dexamethasone, an agent that inhibits cytokine-stimulated release of IL-6, MMP, and PGE<sub>2</sub> by synoviocytes (30, 43), inhibited PTHrP release as well as this morphologic transformation, thus suggesting that increased PTHrP gene expression may be one additional component of a programmed change in gene expression that occurs when synoviocytes are activated to secrete inducible mediators of bone resorption. Similarly, IFN- $\gamma$ , an agent that also inhibits cytokine-stimulated release of bone resorbing agents in synoviocytes (34, 44), inhibited cytokine-stimulated PTHrP release, a result that is also consistent with previous studies by Funk et al. demonstrating IFN- $\gamma$  inhibition of cytokine-stimulated PTHrP production by splenic stromal cells *in vivo* (5, 6). Therefore, just as induction of NH<sub>2</sub>-terminal PTHrP by TNF and IL-1 supports a role for PTHrP as a potential mediator in the pathogenesis of RA, inhibition of PTHrP release by dexamethasone and IFN- $\gamma$ , two agents with some clinical efficacy in the treatment of RA (45, 46), also underscores the potentially destructive, proinflammatory role that PTHrP may play in this disease.

Confirming the proinflammatory potential of synovial NH<sub>2</sub>-terminal PTHrP in RA, PTHrP(1–34) stimulated IL-6 release from cultured synoviocytes. Thus, PTHrP, in addition to its well-described catabolic effects in cartilage and bone, may also act in an autocrine fashion within the synovium to stimulate the production of inflammatory mediators of bone resorption, such as IL-6, a proinflammatory cytokine expressed in increased amounts in the rheumatoid synovium that is also a major mediator of PTHrP-induced bone resorption (12–14, 18).

As in the host response to infection, TNF is thought to be at the apex of the cascade of inflammatory mediators induced in RA, with IL-1 induction occurring distal to TNF, and IL-6 induction distal to TNF and IL-1 (13). However, both clinical trials and animal studies evaluating the effect of TNF blocking antibodies and other anticytokine agents on disease activity

suggest that multiple agents within the rheumatoid cytokine cascade may need to be targeted to prevent disease progression and, in particular, joint destruction (47). The data presented here suggest that PTHrP induction occurs at a later point in the cytokine cascade, distal to activation of TNF and IL-1, but proximal to IL-6, an important proinflammatory cytokine whose production is, in turn, stimulated by PTHrP. Therefore, PTHrP may be one additional downstream target to be considered for anticytokine therapy in RA.

While these studies have focused on increased expression and local effects of PTHrP in the rheumatoid synovium, it is possible that levels of expression of this peptide, which is produced constitutively in cartilage and bone, may be similarly upregulated in areas of bone and cartilage adjacent to sites of invasion by TNF- and IL-1-producing synovial tissue (3, 48). Preliminary investigation of PTHrP expression in rat streptococcal cell wall-induced arthritis, an animal model of inflammatory arthritis (49), suggests that this may indeed be the case (Funk, J., unpublished observation). Thus, a proinflammatory cytokine-mediated increase in PTHrP expression at multiple sites throughout the joint may lead to increased joint destruction via paracrine or autocrine effects in bone, cartilage, and/or synovium in inflammatory arthritis.

In summary, these data suggest a potential role for locally produced NH<sub>2</sub>-terminal PTHrP in the pathogenesis of RA. TNF and IL-1, two locally produced cytokines that are thought to be major mediators of joint destruction, stimulate increased production of NH<sub>2</sub>-terminal PTHrP by synoviocytes, cells which directly invade cartilage and bone in RA. Increased levels of synovial NH<sub>2</sub>-terminal PTHrP may then contribute to joint destruction in RA through direct effects on cartilage or bone, or, indirectly, via the induction of mediators of bone resorption in the tumor-like synovium. When viewed in the context of our previous studies of PTHrP expression during endotoxemia, these results also support the postulate that PTHrP, like TNF or IL-1, is a common component of the cytokine cascade mediating the host response during inflammation. Therefore, PTHrP-mediated bone resorption may not only be an unfortunate consequence of systemic elevations in tumor-derived peptide, but may also be an important component of local cytokine regulation of bone metabolism in a variety of clinical settings.

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