Production of parathyroid hormone-related peptide by synovial fibroblasts in human osteoarthritis

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Abstract Synovial fibroblasts from patients with osteoarthritis in culture produced parathyroid hormone-related peptide (PTHrP) on treatment with phorbol ester (TPA) in a dose- and time-dependent manner. The levels of PTHrP immunoreactivity in the conditioned medium of synovial fibroblast cultures were measured using specific PTHrP antibody. The maximum production was obtained at a concentration of 10^{-8} M and 24 h after TPA treatment. But sensitivity to TPA of synovial fibroblasts differed among four patients from slight to marked. PTHrP production was also induced with inflammatory cytokines, such as 1 ng/ml of IL-1α, IL-1β, IL-6 and TNF-α, and 10^{-6} M prostaglandin E_2, after 24 h treatment. The expression of PTHrP was confirmed by reverse-transcriptase polymerase chain reaction. Since the synovial fibroblasts isolated from osteoarthritic patients produce high levels of IL-6 and IL-8, typical cytokines produced in synovial fibroblasts, production of PTHrP may provide new insight into the pathophysiology of joint disorder.

Key words: Parathyroid hormone-related peptide; Osteoarthritis; Synovial fibroblast; Cytokine; Phorbol ester; Human

1. Introduction

The parathyroid hormone-related peptide (PTHrP) was first identified as a tumor-derived peptide responsible for a syndrome known as humoral hypercalcemia of malignancy [1,2]. Although the biological function of PTHrP is still unclear, it is thought that this peptide plays an important role in the development [3] and pathophysiology [4,5] of numerous diseases. Widespread abnormalities in endochondral ossification and skeletal development were shown in PTHrP-deficient mice [3,6]. Recently it has been reported that synovial fluids from patients with osteoarthritis and rheumatoid arthritis contain high levels of PTHrP [7]. Expression of PTHrP in normal and abnormal cartilage tissue from patients with osteoarthritis or rheumatoid arthritis, using immunohistochemistry and in situ hybridization, was also reported [8]. Furthermore, many kinds of cells in arthral tissue, such as osteoclasts and osteoblasts of deteriorating bony tissue, synovial cells in the superficial layer, macrophages in the deeper layer and endothelial cells in blood vessels, were demonstrated to express PTHrP by immunohistochemistry [9]. However, it has not yet been determined whether PTHrP is actually produced in synovial cells established from synovial membrane in culture. Synovial fibroblasts, abundant components of the synovial membrane, proliferate markedly in arthritis and cause hypertrophy. Then, synovial fibroblasts isolated from synovial membrane of osteoarthritis were established in culture, and examined for PTHrP production in the presence or absence of activating factors such as phorbol ester, inflammatory cytokines and prostaglandin E_2 (PGE_2). In this study we demonstrate that production of PTHrP was induced in synovial fibroblasts with not only phorbol ester TPA, but also inflammatory cytokines and PGE_2. PTHrP influences the proliferation of lymphocytes [10], and the N-terminal peptide portion (1–34) acts as a stimulator of in vivo bone resorption when applied intermittently to neonatal mice [11]. Our findings suggested that PTHrP produced by synovial fibroblasts plays a novel role as a paracrine/autocrine factor in the pathophysiology of osteoarthritis.

2. Materials and methods

2.1. Patients

Osteoarthritis was diagnosed and classified on the basis of radiographic findings. Radiographs of osteoarthritic patients showed joint-space narrowing, sclerotic changes, and erosion of grade III. Synovial tissues were obtained from four female patients (age range 67–76) when they underwent the arthroplasty operation for severe inflammatory or destructive lesions in the knee. Informed consent was obtained from each patient.

2.2. Isolation and culture of synovial fibroblasts

The synovial cells were isolated according to the method of Takayanagi et al. [12]. In brief, the synovial tissue was dissected away, washed twice with RPMI 1640 (Gibco BRL, Gaithersburg, MD, USA), and finely minced. The minced tissue was incubated in RPMI 1640 containing 1 mg/ml collagenase (Wako Pure Chemicals, Osaka, Japan) and 0.15 mg/ml DNase I (Sigma, St. Louis, MO, USA) and incubated with shaking for 90 min at 37°C. The cell suspension was filtered through a 70 μm cell strainer (Becton-Dickinson, Franklin Lakes, NJ). The cell suspension (3 ml) was layered on 4 ml of Ficoll/Paque (Pharmacia Biotech, Uppsala, Sweden) and centrifuged at $400 \times g$ for 30 min at 20°C. The interface layer was resuspended in 4 volumes of RPMI 1640 and washed three times by centrifugation at $250 \times g$ for 10 min. The cells were finally suspended in α-minimum essential medium (α-MEM) (Irvine Scientific, Santa Ana, CA) containing 20% heat-inactivated horse serum (Gibco BRL, Gaithersburg, MD) and cultured in a 5% CO_2 incubator. Medium was changed every 3 days. When cells reached confluence, those that dispersed after

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agitating with 0.02% trypsin and 0.05% EDTA in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) for 5 min were transferred to new plastic dishes in a split ratio of 1:2 or 1:4. The culture medium was replaced two times each week. More than seven passaged cells (7 population doubling levels, PDL) were used for subsequent experiments. This cell fraction consisted of only fibroblasts, with no dendritic or monocytic cells.

2.3. Treatment with cytokines and other stimulants

The synovial fibroblasts at 7–12 PDL were treated with trypsin/EDTA solution for 5 min to detach them from dishes and collected by centrifugation at 250×*g* for 10 min. The detached cells were plated onto 12 well multiwell dishes at 2×10^5 cells/well with 1.0 ml of α-MEM containing 20% horse serum. When cells reached confluence, they were incubated with various concentrations of phorbol 12-myristate 13-acetate (TPA) (Sigma), 1 or 10 ng/ml of recombinant human cytokines such as interleukin (IL)-1α, IL-1β, IL-6 and tumor necrosis factor (TNF)-α (Genzyme Diagnostics, Cambridge, MA, USA), and 10^{-6} or 10^{-5} M of PGE_2 (Wako Pure Chemicals) for 24 h after replacement of the culture medium with α-MEM supplemented with 5% horse serum. The PTHrP level in the conditioned medium was measured directly by immunoradiometric assay (IRMA). The amount of DNA in each well was measured by the method of Burton [13] after extraction with hot 5% perchloric acid.

2.4. Measurement of PTHrP, L-6 and IL-8 in the conditioned medium

PTHrP concentrations in the conditioned medium was measured by two-site IRMA method (Allegro PTHrP, Nichols Institute Diagnostics, Capistrano, CA, USA) using two kinds of polyclonal antibodies against human PTHrP (1–86). The two-site IRMA method was performed with ^{125}I-polyclonal antibody against N-terminal residues (1–40) of human PTHrP and with biotin-polyclonal antibody against C-terminal residues (60–72) of human PTHrP by the addition of avidin-coated beads. The sensitivity of this assay is 0.2 fmol/ml. The inter-assay coefficient of variation is 7.67–16.89%. Human intact PTH(1–84), PTH fragment (1–34), N-terminal fragments of PTHrP (1–40), and C-terminal fragment of PTHrP (109–141) were not detected. Details have been described previously [14]. Determination of human IL-6 and IL-8 was by enzyme linked immunoassay (ELISA), using Perseptive Biosystems (Framingham, MA, USA). The sensitivity of this assay is 1 pg/ml.

2.5. Reverse transcriptase PCR (RT-PCR)

RT reaction and PCR amplification were performed using the RNA LA PCR kit (Ver. 1.1, Takara Biomedicals, Osaka, Japan). 1 μg of total RNA from synovial fibroblasts (T-12 cells) at 10 PDL treated with stimulants for 24 h was hybridized to oligo dT-adaptor primer, and the RT reaction was carried out using AMV reverse transcriptase XL (Life Sciences, FL, USA) for 1 h at 42°C. The synthetic forward primer (5′-CTGGT TCAGC ATGGG AGGGTC-3′) and the reverse primer (5′-GTTAG GGGAC CACCT CCGAGGT-3′) were designed to amplify a 231 bp fragment. The following PCR profile was used as described by Li et al. [15]: denaturation at 94°C for 60 s, reannealing at 60°C for 45 s, extension at 72°C for 60 s, 45 cycles. Amplified products were separated by electrophoresis on a 2.0% agarose gel. GAPDH primers were used to control cDNA samples.

3. Results

3.1. PTHrP production in synovial fibroblasts

The synovial fibroblasts, T-2, T-12, T-17, and T-21 cells, established from synovial membrane of four patients with osteoarthritis were treated with various concentrations of phorbol ester TPA at confluence. Production of PTHrP in the culture medium was measured by the two-site IRMA method for human PTHrP (1–86). The production maximized at 10^{-8} M TPA treatment and then gradually decreased with increasing concentration in all cell strains except T-17 cells (Fig. 1). The induction of PTHrP with TPA differed among cell strains. T-17 cells were scarcely induced by the treatment, whereas T-12 cells were markedly induced. These treatments did not affect cell morphology or viability. Since the fibroblasts were subcultured more than seven times for 6–8 weeks, dendritic cells, monocytes, and other blood cells derived from synovial membrane disappeared, and only the fibroblasts remained. Further investigations were performed using T-12 cells. Production of PTHrP was induced 6 h after treatment, and increased in a time-dependent manner up to 24 h. Incubation beyond this did not induce production further (Fig. 2). Induction did not occur under low serum conditions, as in α-MEM supplemented with 0.2% horse serum (data not shown). Culture medium alone did not react with the antibody used in this assay system.

3.2. Cytokine-induced PTHrP

Whether inflammatory cytokines such as IL-1α, IL-1β, IL-6, and TNF-α induce PTHrP in synovial fibroblasts was investigated. Both IL-1α and IL-1β induced PTHrP in a dose-dependent manner. The amount of PTHrP induced by IL-1α was equal to that by 10^{-9} M TPA. But IL-6 and TNF-α slightly enhanced the production. High concentrations of IL-6 and TNF-α may be required for efficient induction. PGE_2, which has a strong inflammatory effect and is generally produced in inflammatory tissues, also induced PTHrP in a dose-dependent manner (Fig. 3). Expression of mRNA of PTHrP in the fibroblasts was confirmed by RT-PCR. As shown in Fig. 4, untreated cells also expressed the mRNA, although TPA or IL-1α treatment only slightly enhanced the mRNA level. Forskolin and TNF-α did not enhance PTHrP expression. The level of GAPDH expression did not vary among samples.

These results suggest that synovial fibroblasts from patients with osteoarthritis produce PTHrP during inflammation process.

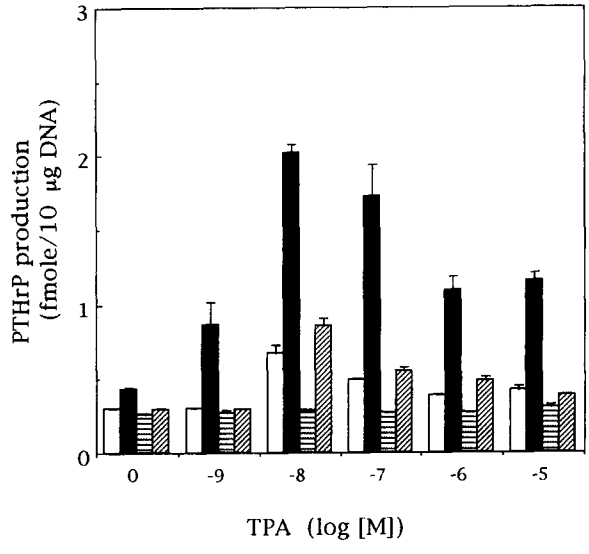

Fig. 1. TPA-induced PTHrP production in synovial fibroblasts. Synovial fibroblasts established from four patients with osteoarthritis in culture at 7–12 PDL were plated to 12 well multiwell plates. Confluent cells were incubated with various concentrations of phorbol ester TPA in 0.5 ml of α-MEM supplemented with 5% horse serum for 24 h. The PTHrP released in the conditioned medium was directly assayed by the two-site IRMA method for human PTHrP (1–86). The values represent the mean ± S.E.M. ($n=3$). The DNA content per well was 10.5–19.5 μg. White bars: T-2 cells (9 PDL); black bars: T-12 cells (12 PDL); horizontally hatched bars: T-17 cells (7 PDL); diagonally hatched bars: T-21 cells (8 PDL).

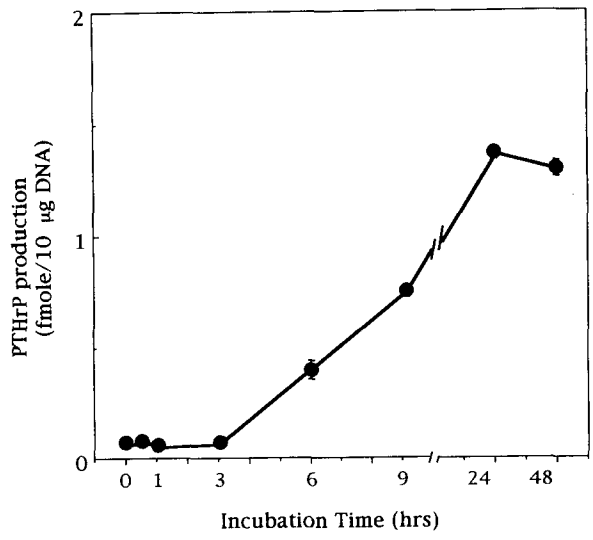

Fig. 2. Time-dependent increase of PTHrP with TPA treatment in synovial fibroblasts. Confluent T-12 cells were incubated with 10^{-8} M TPA for the indicated times. The amount of PTHrP released in the medium was measured as described in Fig. 1.

3.3. *Production of IL-6 and IL-8 by synovial fibroblasts*

To identify fibroblast phenotype, production of IL-6 and IL-8 in conditioned medium (see Fig. 1) was measured by ELISA. These cells produced detectable amounts of IL-6 and IL-8 without any stimulants. However, the production was markedly induced with TPA treatment. The induction rate for IL-6 and IL-8 was more than that for PTHrP, with the same tendency among three cell strains (Table 1). T-12 cells were tremendously sensitive to TPA treatment in comparison with T-2 and T-17 cells. In T-17 cells, PTHrP production was not induced with TPA treatment, but IL-6 and IL-8 productions were induced at the same level as in T-2 cells. T-12 cells also produced a large quantity of prostaglandin E_2 (data not shown) as reported previously [16]. IL-1β and TNF-α were not detected (data not shown).

Table 1
IL-6 and IL-8 production in synovial fibroblasts

Cell strain	Treatment with TPA (M)	Cytokine production (pg/ml)	
		IL-6	IL-8
T-2	0	683 ± 7	1651 ± 87
	10^{-9}	2678 ± 173	41804 ± 4021
	10^{-8}	9131 ± 503	81547 ± 12323
	10^{-7}	7932 ± 405	53407 ± 4504
	10^{-6}	7703 ± 978	44618 ± 5615
	10^{-5}	6050[a]	40438[a]
T-17	0	180 ± 21	251 ± 56
	10^{-9}	631 ± 166	1483 ± 859
	10^{-8}	1574 ± 100	12056 ± 3398
	10^{-7}	1530 ± 137	7998 ± 530
	10^{-6}	1244 ± 122	5553 ± 3309
	10^{-5}	1459 ± 217	6941[a]
T-21	0	501 ± 28	180 ± 22
	10^{-9}	1570 ± 400	2456 ± 782
	10^{-8}	1912 ± 95	10138 ± 3964
	10^{-7}	2183 ± 94	7839 ± 590
	10^{-6}	1948 ± 94	3449 ± 567
	10^{-5}	2130 ± 117	3848[a]

The levels of IL-6 and IL-8 released in the medium described for Fig. 1 were measured by EIA. Values represent the mean ± S.E.M. ($n = 3$). [a]Indicates the value from one sample.

4. Discussion

This study presents the first finding of PTHrP production by synovial fibroblasts from patients with osteoarthritis in vitro. The accumulation of PTHrP in synovial fluid has been reported by Kohno et al. [7] and Okano et al. [8]. The synovial fluid level of the C-terminal region of PTHrP (C-PTHrP) was markedly higher in rheumatoid arthritis compared with control and osteoarthritis groups, while no differences in circulatory C-PTHrP in serum were present among the three groups. In contrast, the synovial fluid level of the N-terminal region of PTHrP (N-PTHrP), which has a stimulatory effect on bone resorption [17], was marginally higher in osteoarthritis. Moreover, immunoreactivity to PTHrP (38–64, mid-portion) antibody was more frequently identified in osteoarthritic cartilage (specimens) than rheumatoid arthritic specimens and control subjects [8]. PTHrP produced in arthral tissue likely has important roles in osteoarthritis and subsequent cartilage degeneration and bone destruction. In this study we found that inflammatory cytokines and PGE_2 reported in synovial fluid [16] induced PTHrP production by synovial fibroblasts. Our results suggested PTHrP production by synovial fibroblasts contributes to the inflammatory process in arthral tissue. Synovial fibroblasts in culture did not decrease PTHrP production during passage from 7 PDL to 12 PDL, but four strains donated by different individuals produced levels of PTHrP that varied widely. Since we used the synovial membranes of surgical waste, the inflammation was chronic, not acute, as shown by the low basal production level (Fig. 1, Table 1). The IL-6 and IL-8 productions in synovial fibroblasts [18] were higher than in human skin fibroblasts at the basal level (data not shown), thus the fibroblasts established here appear to maintain the characteristics of synovial fibroblasts. Interestingly, these cells produced M-CSF (macrophage colony stimulating factor) and PGE_2, and scarcely produced IL-1 and TNF-α (data not shown). Phorbol

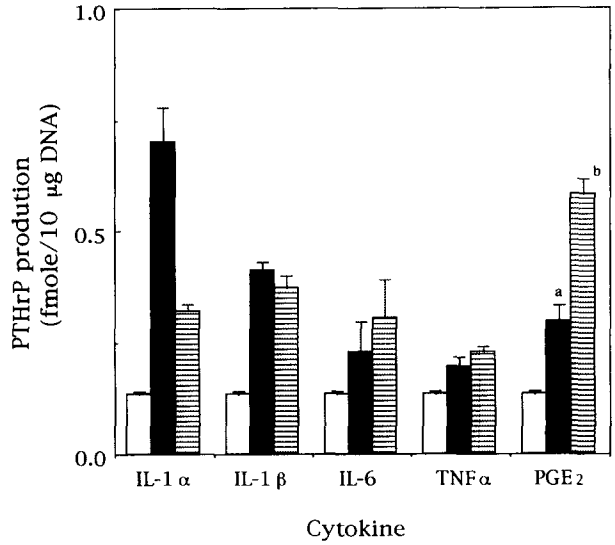

Fig. 3. Cytokine- and PGE_2-induced PTHrP production by synovial fibroblasts. Confluent T-12 cells were incubated with 1 ng/ml (black bars) or 10 ng/ml (hatched bars) of IL-1α, IL-1β, IL-6 and TNF-α for 24 h. The PTHrP released in the conditioned medium was measured as described in Fig. 1. In the case of PGE_2, 10^{-5} M (black bar) or 10^{-6} M (hatched bar) PGE_2 was used. White bars: untreated control cells.

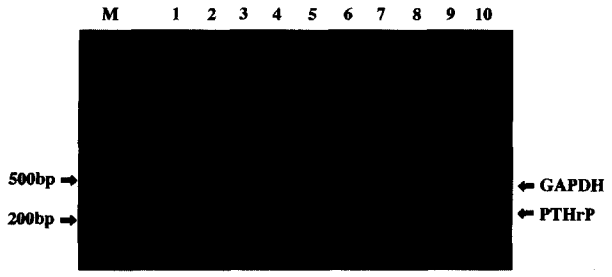

Fig. 4. Expression of PTHrP in T-12 cells as assessed by RT-PCR. An ethidium bromide-stained 2% agarose gel is shown after electrophoretic separation of RT-PCR products. Confluent T-12 cells were incubated with 10^{-8} M TPA (lanes 2, 7), 10^{-5} M forskolin (lanes 3, 8), 10 ng/ml IL-1α (lanes 4, 9) and 10 ng/ml TNF-α (lanes 5, 10) for 24 h. Untreated control cells are shown in lanes 1, 6. Total RNA was used for RT-PCR analysis. Lanes 1–5 show PTHrP expression (231 bp), and lanes 6–10 show GAPDH expression. Lane M: DNA size markers.

ester TPA, a protein kinase C activator, induced PTHrP production, as reported in rat articular cartilage [19] and mammary epithelial cells [20], but 10^{-8} M phorbol ester TPA only slightly changed the level of PTHrP mRNA. In our experiment, the level of PTHrP mRNA in RT-PCR changed little on addition of TPA or IL-1α, which contrasts with the enhancement in the conditioned medium. It is difficult to compare PTHrP mRNA levels among samples quantitatively by the RT-PCR analysis used here.

Our findings suggest that PTHrP production is induced in synovial fibroblasts in the inflammatory state, at the protein and mRNA level.

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