IL-1-mediated expression of membrane type matrix-metalloproteinase in rheumatoid osteoblasts

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Abstract

Objective
To determine whether the activation of metalloproteinases can be achieved by the interaction of the inflammatory cytokine, IL-1β, with rheumatoid osteoblasts.

Results
MMP-2 is not secreted by rheumatoid osteoblasts as a proenzyme; however, IL-1β stimulation induced the secretion of MMP-2 as an active form from rheumatoid osteoblasts. This MMP-2 activating activity was detected significantly in IL-1β-stimulated rheumatoid osteoblasts. In support of this result, IL-1β stimulation induced the expression of membrane type matrix-metalloproteinase (MT-MMP), a newly-identified MMP-2 specific activator, in rheumatoid osteoblasts.

Conclusion
These results suggest that IL-1β induces MMP-2 activation in part by up-regulating MT-MMP expression and represents a new mechanism for cytokine-mediated articular destruction in RA.

Key words
Matrix metalloproteinase, cytokine, interleukin-1 beta (IL-1β), rheumatoid arthritis, osteoblast.

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Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by marked inflammation of multiple joints (1). The inflamed joints, in which pannus develops, are rich in inflammatory cells including activated lymphocytes (2-5). These cells produce several kinds of soluble factors including cytokines, and high concentrations of various cytokines have been detected in RA synovium (6-8). A previous study showed that interleukin 6 (IL-6) inhibits collagen synthesis and alkaline phosphatase (ALP) activity in osteoblasts in vitro (9). It was also recently reported that osteoblasts adhered to activated T cells through adhesion molecules, and these cellular interactions increased the cytokine production by osteoblasts (10). Although these results suggest the importance of the modulation of osteoblast function in articular destruction in inflammatory conditions such as RA, it had not been elucidated whether the interaction of inflammatory cytokines and rheumatoid osteoblasts directly contributed to the destruction of inflamed joints, especially extracellular matrix degeneration. Rheumatoid synovial fibroblasts produce a number of MMPs including MMP-1, 2, 3, and 9 (11-13). Increased concentrations of these MMPs were demonstrated in synovial fluids from patients with RA (14-16). Cytokines produced by synovial inflammatory cells are thought to induce the secretion of MMPs from rheumatoid synovial cells (12, 16, 17). It had not been elucidated exactly how osteoblasts contributed to MMP secretion in inflamed joints, although a number of reports concerning the production of MMPs by osteoblasts has been gradually increasing (18, 19).

To identify the activation mechanisms for MMPs induced by inflammatory cytokines on rheumatoid osteoblasts of inflamed joints, we examined whether IL-1β increased the gelatinase expression in rheumatoid osteoblasts by a gelatin zymography, and whether IL-1β induced the expression of MT-MMP in rheumatoid osteoblasts using an immunoblot analysis and a RT-PCR method.

Materials and methods

Materials

Mouse anti-human MT-MMP-1 monoclonal antibody and mouse anti-human MMP-2 antibody were purchased from Fuji Chemical (Takaoka, Japan). Human recombinant IL-1β was generously provided by Dainihon Chemical Co. (Osaka, Japan). Human recombinant tumor necrosis factor-alpha (TNF-α) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

Cell culture

Human osteoblastic cell line MG63 and human primary osteoblast cells were used in the present study. Human primary osteoblasts were obtained from patients with RA and osteoarthritis (OA) who underwent total hip or knee joint replacement. Informed consent for the subsequent experiments was obtained from each patient.

The bones were minced aseptically, then dissociated enzymatically 4 times with collagenase (4.0 mg/ml, Sigma) in RPMI 1640 for 30 min at 37°C. The obtained cells were plated in culture dishes and allowed to adhere. To eliminate non-adherent cells from the osteoblast preparation, the plated cells were cultured for 18 hr with RPMI 1640, supplemented with 10% FCS at 37°C in humidified 5% CO2 in air. Cells were then washed extensively with phosphate buffered saline solution (PBS). Adhering osteoblasts were removed by adding trypsin-EDTA and washed with PBS containing 2% FCS. Osteoblasts collected at the 3rd or 4th passage were used for subsequent experiments.

The production of osteocalcin from isolated osteoblasts was determined before the present study using an ELISA assay, and the alkaline phosphatase (ALP) activity of these cells was determined by the method described by Lowry et al. (20). In addition, isolated osteoblasts were positively stained when the cells were incubated with 2-amino-2-methyl-1,3-propandiol buffer (Wako Pure Chemical Industries, Osaka, Japan) containing naphthol AS-MX phosphate and fast blue RR salt (data not shown, Sigma Chemical Co., St. Louis, MO) (21). Cells were plated in 6-well culture plates (Costar, Cambridge, MA) for 24 hrs, then washed 3 times with PBS before serum-free RPMI 1640 medium supplemented with various concentrations of IL-1β was added.
**Gelatin zymography**

Culture media were incubated at 37°C for 20 min in sodium dodecyl sulphate (SDS) sample buffer without a reducing agent and then electrophoresed on 8% polyacrylamide gels containing 1 mg/ml gelatin at 4°C. After electrophoresis, the gels were washed in 2.5% Triton-X 100 to remove SDS, and incubated with 50 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl, 10 mM CaCl₂ and 0.02% NaN₃ for 16 hrs at 37°C and stained with 0.1% Comassie Brilliant Blue R250.

**Immunoblot analysis**

Osteoblast monolayers were rinsed with Tris-buffered saline (10 mM Tris-HCl pH 7.5, 150 mM NaCl), scraped in ice-cold Tris-buffered saline and sonicated on ice in the presence of proteinase inhibitors (0.1 mM aprotinin, 1 mM leupeptin, 1 mM EDTA and 1 mM pepstatin). An identical amount of proteins (40 μg) for each lysate was subjected to 8% SDS-PAGE. Also, osteoblast-conditioned media were dissolved on SDS-PAGE.

Proteins were transferred to nitrocellulose membrane. The filters were blocked for 1.5 hr using 5% non-fat dried milk in TBS (50 mM Tris, 0.15 M NaCl, pH 7.5) containing 0.1% Tween 20 (TBS-T), washed with TBS-T, and incubated at room temperature for 2 hr in a 1:250 dilution of mouse anti-MT-MMP monoclonal antibody or a 1:250 dilution of mouse anti-MMP-2 monoclonal antibody. Filters were later washed with TBS-T and incubated with a 1:1000 dilution of donkey anti-mouse IgG antibodies, coupled with horseradish peroxidase. The enhanced chemiluminescence (ECL) system (Amersham) was used for detection. Filters were subsequently exposed to film for 15 sec and the latter was processed.

**RNA preparation and RT-PCR**

Total RNA was extracted from MG63 cells and human primary osteoblasts using Isogen (Nippon gene, Co. Ltd. Tokyo, Japan). First-strand cDNA was synthesized by reverse transcription at 37°C for 90 min in a 20 μl reaction mixture containing oligo dT primer by MuLV reverse transcriptase (Gibco BRL, Gaithersburg, ND) from 1 μg of total RNA. After heating at 95°C for 5min to denature the proteins then cooling to 4°C, cDNA was amplified in a total volume of 20 μl with 1U Taq DNA polymerase (Gibco BRL), 1 mM of both primers, and Taq polymerase buffer containing 1.5 mM MgCl₂ with 1.5 mM of dNTP. PCR was carried out on a thermal cycler (Perkin Elmer Cetus) using a program of 30 cycles (1min denaturation at 94°C, 1 min annealing at 55°C, 1 min elongation at 72°C and a final extension at 72°C for 10 min). The amplified products were subjected to electrophoresis on 1.5% agarose gels and visualized by UV detection.

To quantify the PCR products comparatively and to confirm the use of equal amounts of the RNAs, we amplified β-actin. Specific primers were as follows (22); MT-MMP-1 (sense) 5’-ATTGAT GCTGCTCTTCTGTCG-3’, MT-MMP-1 (antisense) 5’-GTGAAGACTTCAT CGCTGGC-3’. The predicted fragment size was 182 bp. β-actin (sense) was 5’-GACGAGGGCCCAG AGCAAGAGAG-3’; β-actin (antisense) was 5’-ACGTA CATGGTTGGGTGTG-3’. The predicted fragment size was 236 bp. After amplification, 6μl of each PCR reaction mix was subjected to electrophoresis on 1.5% (w/v) agarose gels with ethidium bromide (0.5 mg/ml). Results

Osteoblasts isolated from RA patients were seeded in RPMI 1640 medium supplemented with 10% FCS and cultured for 48 hr before being switched to serum-free medium. Osteoblasts were then incubated for another 48 hr in the presence or absence of IL-1β (0.1U/ml, 1U/ml, 10U/ml) and TNF-α (1U/ml, 10U/ml, 100U/ml). Conditioned media were collected and electrophoresed on gelatin substrate gels to analyse the gelatin-degrading activity. Gelatin zymography of media from IL-1β-stimulated osteoblasts revealed the induction of gelatinase activity corresponding to an electrophoretic mobility of 66kDa and 62kDa (Fig. 1). Molecular masses determined from zymograms are those of non-reduced proteins. Under these conditions the 72kDa progelatinase has an apparent molecular mass of 66 kDa (35). Activation of progelatinase A (MMP-2) involves autocatalytic cleavage to yield an active form of MMP-2. Under the 66 kDa band gelatinolytic bands were detected in IL-1β-stimulated osteoblast-conditioned media, which should be active forms of MMP-2. TNF-α induced gelatinolytic activity in the cultured media of osteoblasts, but not of IL-1β. We also determined whether IL-1β could induce gelatinase activity in the cultured media of osteoblasts from joints of OA.
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(Fig. 2). Osteoblasts of OA responded to IL-1β less than RA osteoblasts. IL-1β did not induce as strong a band on gelatin substrate gel and could not induce an active form of MMP-2 definitely.

To confirm whether IL-1β stimulates the production of MMP-2, Western blot analysis was carried out using anti-MMP-2 mAb. MMP-2, which migrates as a 72 kDa band, was faintly detected in media conditioned by non-treated osteoblasts. Incubation of osteoblasts with IL-1β induced the secretion of 72 kDa MMP-2 (data not shown). These results demonstrated that activation of MMP-2 occurred in RA osteoblast cultures when the cells were stimulated by the inflammatory cytokine, IL-1β.

Recently, Sato et al. cloned the cDNA encoding a new membrane-type metalloproteinase (MT-MMP) which induces the specific activation of pro-MMP-2. So we examined whether osteoblasts express MT-MMP by Western blot analysis. IL-1 beta stimulation enhanced MT-MMP expression in rheumatoid osteoblasts (Fig. 3A). Though OA osteoblasts were incubated with IL-1β as well as rheumatoid osteoblasts, the enhanced effect of IL-1β on the expression of MT-MMP-1 was weak in OA osteoblasts (Fig. 3B). Furthermore, we determined whether TNF-α induced the expression of MT-MMP-1 in rheumatoid osteoblasts. TNF-α induced the expression of MT-MMP-1 in rheumatoid osteoblasts. However, TNF-α-induced MT-MMP-1 expression was weak compared to IL-1β (Fig. 4).

To examine the regulation of MT-MMP-1 expression, MG63 and human primary osteoblasts were treated with IL-1β, then the mRNA was extracted. MT-MMP-1 mRNA expression was evaluated by RT-PCR. MT-MMP-1 mRNA was faintly detected in serum-starved cells, and its expression was strengthened when the cells were stimulated by IL-1β (Figs. 5 and 6).

Discussion

The mechanism of articular destruction in RA has not been identified; however, it has been reported that rheumatoid synovial fluids contain various proteinases which may contribute to the cartilage damage (23). In addition, elevated levels of MMPs (MMP-1, 2, 3, 9), which induce extracellular matrix degradation, have been observed in joint fluid and synovium from patients with RA (14-16, 24-30). The MMP-1 concentration in RA synovial fluid correlates positively with the degree of synovial inflammation, and increased with increasing numbers of cells identified as expressing MMP-1 in synovial tissue (26, 27). The concentration of MMP-3 was elevated in synovial fluids from patients with RA and the increased level of MMP-3 in synovial fluid may reflect an elevated matrix degrading activity due to joint inflammation (24-26).

Futhermore, inflammatory cytokines, such as IL-1 and TNF-α, are linked to
abnormal cartilage and bone loss in a variety of pathological conditions and the production of MMPs by rheumatoid synovial fibroblasts (17, 31). Panagakos et al. (18) have reported that TNF-α induced MMP-1, 2, 9 production by human osteoblastic cells in monolayer cultures. These results suggest that inflammatory cytokines increase pathological bone turnover by enhancing the rate of transcription of proteases capable of degrading the nonmineralized osteoid layer and decelerating the maturation of the ECM formed by osteoblasts. MMPs are secreted in a latent inactive form and converted by the proteolysis of other enzymes or MMPs (32-35). The Mr 72000 gelatinase A (pro-MMP-2) is a MMP, which has an ability to degrade many connective components including collagens. It was shown that MMP-2 was secreted from rheumatoid synovial fibroblasts (13, 35). However, the process responsible for the pro-MMP-2 activation, which may directly contribute to the degradation of rheumatoid cartilage components, is not well understood.

In the present study, we examined the role of the inflammatory cytokine, IL-1β, in the activation of pro-MMP-2 secreted from rheumatoid osteoblasts. We found that MMP-2 is not produced by rheumatoid osteoblasts, and IL-1β stimulation induced the secretion of MMP-2 with the conversion of pro-MMP-2 to an active form of MMP-2.

Sato et al. identified a 63 kDa membrane-type matrix metalloproteinase (MT-MMP) and reported that the expression of MT-MMP induced the specific activation of pro-MMP-2 (36-38). We examined this newly identified MT-MMP, and found that it is induced in osteoblasts by IL-1β stimulation. Previous reports suggest that the putative MMP-2 activator is localized to the cell surface and up-regulated by Con A or TPA (39, 40). The expression of MT1-MMP in COS-1 cells induces cell-surface binding of progelatinase A which is consequently processed to an active form (38). Yu et al. demonstrated that Con A induced the up-regulation of MT-MMP expression in human breast cancer cells (41). Yoshizaki et al. (42) also suggested that increased expression of the MT1-MMP is involved in the activation of pro-MMP2 in head and neck squamous cell carcinoma. It was hypothesized that exogenous MMP-2 activation by MT-MMP on the malignant cells is critical for tumor cell invasion and metastasis (36, 43). Recently, it was reported that MT1-MMP was expressed in human osteoarthritic cartilage (44, 45). Intriguingly, the expression of MT1-MMP was induced by treatment with inflammatory cytokines in osteoarthritic cartilage. Our results suggest a novel role for IL-1β inducing the MMP-2 activation possibly mediated by MT-MMP in osteoblasts during inflammatory joint disease, RA. The activation of MMP-2 correlated with the ECM degradation. Sato et al. identified MT1-MMP in rabbit osteoclasts (46).

**Fig. 4.** Expression of membrane type matrix metalloproteinase (MT-MMP-1) induced by TNF-α in rheumatoid osteoblasts. Rheumatoid osteoblasts were incubated with TNF-α at concentrations of 0 U/ml (lane 1), 1 U/ml (lane 2) 10 U/ml (lane 3), and 100 U/ml (lane 4) for 48 hrs. Cellular lysates were analyzed by immunoblot using anti-MT-MMP-1 mAb. Experiments were repeated at least 3 times.

**Fig. 5.** RT-PCR analysis of MT-MMP transcripts in IL-1β-stimulated MG63. MG63 were incubated with 10 U/ml IL-1β for the period indicated. Total RNA was then isolated and analyzed by RT-PCR using random labeled cDNA probes for membrane-type matrix metalloprotease (MT-MMP-1). A 550-bp RT-PCR product of the MT-MMP-1 mRNA was visualized with ethidium bromide. The β-actin mRNA level was used as an internal standard. Experiments were repeated at least 3 times.

**Fig. 6.** RT-PCR analysis of MT-MMP transcripts in IL-1β-stimulated rheumatoid osteoblasts. Rheumatoid osteoblasts were incubated with 10 U/ml IL-1β for the period indicated. Total RNA was then isolated and analyzed by RT-PCR using random labeled cDNA probes for membrane-type matrix metalloprotease (MT-MMP-1). A 550-bp RT-PCR product of the MT-MMP-1 mRNA was visualized with ethidium bromide. The β-actin mRNA level was used as an internal standard. Experiments were repeated at least 3 times.
We propose a potential mechanism for the pro-MMP-2 activation in rheumatoid articular destruction. IL-1β-stimulated osteoblasts may convert the pro-MMP-2 to the active form by expressing MMP-2 activator, MT-MMP, on the cell surface.

In rheumatoid inflamed joints, inflammatory cytokines such as IL-1, TNF-α, and transforming growth factor (TGF) are secreted by activated macrophages, monocytes and synovial fibroblasts (47, 48). The interaction of these cytokines and inflammatory cells may result in the perpetuation of the articular inflammation and destruction.

IL-1β has been considered as a candidate for the cytokine responsible for the destruction of the joints and periarticular osteoporosis. We suggest that this cytokine confers on the osteoblasts an enhanced ability to degrade cartilage or bone matrix components by activating MMP-2. Furthermore, our results implicate the IL-1β-mediated MT-MMP expression on osteoblasts as a critical event in the process of MMP-2 activation.

References


