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Regulation of Matrix Metalloproteinases (MMP-2, -3, -9, and -13) by Interleukin-1 and Interleukin-6 in Mouse Calvaria: Association of MMP Induction with Bone Resorption*

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ABSTRACT

Interleukin-1 (IL-1) greatly induces osteoclast formation and stimulates bone resorption of mouse calvaria in culture. In the presence of soluble IL-6 receptor (sIL-6R), IL-6 similarly induces osteoclast formation, but the potency of IL-6 in inducing bone resorption in organ culture is weaker than that of IL-1. To study the differences in bone-resorbing activity between IL-1 and IL-6, we examined the effects of the two cytokines on the induction of matrix metalloproteinases (MMPs). In mouse calvarial cultures, IL-1 markedly enhanced the messenger RNA (mRNA) expression of MMP-13 (collagenase 3), MMP-2 (gelatinase A), MMP-9 (gelatinase B), and MMP-3 (stromelysin 1), which associated with increases in bone matrix degradation. A hydroxamate inhibitor of MMPs significantly suppressed bone-resorbing activity induced by IL-1. Gelatin zymography showed that both pro- and active-forms of MMP-2 and MMP-9 were detected in the conditioned medium collected from calvarial cultures, and IL-1 markedly stimulated both pro- and active-forms of the two gelatinases. IL-6 with sIL-6R also stimulated mRNA expression and biological activities of these MMPs, but the potency was much weaker than that of IL-1. Conditioned medium collected from IL-1-treated calvariae degraded native type I collagen, but ¾- and ¼-length collagen fragments were not detected, suggesting that both collagenases and gelatinases synergistically degraded type I collagen into smaller fragments. In mouse osteoblastic cells, the expression of MMP-2, MMP-3, and MMP-13 mRNAs could be detected, and they were markedly enhanced by IL-1α on days 2 and 5. IL-6 with sIL-6R also induced expression of MMP-13 and MMP-2 mRNAs on day 2, but the expression was rather transient. These results demonstrate that the potency of induction of MMPs by IL-1 and IL-6 is closely linked to the respective bone-resorbing activity, suggesting that MMP-dependent degradation of bone matrix plays a key role in bone resorption induced by these cytokines. (*Endocrinology* **139:** 1338–1345, 1998)

INTERLEUKIN-1 (IL-1) markedly stimulates osteoclastic bone resorption *in vivo* and *in vitro* by enhancing both osteoclast formation and function (1–3). Other cytokines (including IL-6 and IL-11, which transduce their signals through the signal-transducing gp130 chain) also induce osteoclast formation *in vitro* (4–6). Both IL-1 and IL-6 seem to induce osteoclast formation from its progenitors via a mechanism involving osteoblastic cells (6). We reported previously that the presence of soluble IL-6 receptor (sIL-6R) was essential for the osteoclast formation induced by IL-6 in cocultures of mouse bone marrow cells and osteoblastic cells (4). This suggests that osteoblastic cells lack membrane-bound IL-6 receptors under physiological conditions. The potency of IL-6 with sIL-6R in osteoclast formation was equivalent to that of IL-1, but in organ culture systems such as mouse calvarial cultures, bone-resorbing activity of IL-1 was much greater than that of IL-6, even in the presence of sIL-6R. Bone resorption consists of multiple steps, including osteoclast differentiation, activation of osteoclast function, degradation of bone matrix proteins, and bone mineral mobilization. Because IL-1 and IL-6 similarly stimulate osteoclast differentiation, the differences in bone-resorbing activity between IL-1 and IL-6 may be caused by the actions of these cytokines in other processes, such as matrix degradation.

Degradation of the organic matrix in bone depends on the activity of proteolytic enzymes, which consist of 2 major classes: the cysteine proteinase family (such as catepsin K) and the matrix metalloproteinase (MMPs) family (7–11). Over the past years, 18 different mammalian MMPs have been identified. These can be divided into 4 subgroups; collagenases (MMP-1, MMP-8, MMP-13, and MMP-18), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3 and MMP-10), and membrane-type metalloproteinases (MMPs-

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14–17) (12–14). These MMPs are all zinc-dependent endopeptidases with the ability to degrade the organic matrix at physiological pH. Sequence comparisons have revealed that mouse and rat collagenases are homologous to the human collagenase 3 identified by Freije *et al.* (15) and are now referred to as MMP-13. Stromelysins such as MMP-3 not only act as a metalloproteinase but also activate a latent pro-MMP. Therefore, the cooperative effects of collagenases, gelatinases, and stromelysins may be important for MMP-dependent degradation of bone matrix.

Previous reports suggested that MMPs are involved in bone resorption. MMP-13 (collagenase 3) and MMP-2 and MMP-9 (gelatinases A and B) are produced by osteoblasts and/or osteoclasts (8, 10, 16–19). Tezuka *et al.* (20) demonstrated the selective expression of MMP-9 in osteoclasts. Hill *et al.* (21, 22) reported that synthetic inhibitors of collagenase and/or gelatinase prevented bone resorption *in vitro*. It also has been proposed that osteoblast-derived collagenase is responsible for degrading the nonmineralized osteoid layer covering bone surfaces, which is essential for exposing the mineralized matrix to osteoclasts (9, 23). More recently, it was reported that not only denatured, but also native type I collagens could be degraded by MMP-2 (24). MMP-9 failed to degrade native type I collagen. Osteoblasts produce gelatinases such as MMP-2 (16). The regulation of MMP-2 in osteoblasts and involvement of gelatinases in bone resorption, however, are not well understood.

In this study, we examined the regulation of expression of several MMPs by IL-1 and IL-6 at the messenger RNA (mRNA) level in mouse calvarial cultures. Not only MMP-13 (collagenase 3), but also MMP-2 and MMP-9 (gelatinases) and MMP-3 (stromelysin 1), were markedly induced by IL-1 and moderately induced by IL-6. These MMPs may act in concert for the degradation of bone matrix associated with bone resorption.

Materials and Methods

Animals and drugs

Newborn (5 days old) and adult ddy mice (7 weeks old) were obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan). Recombinant human IL-1α was purchased from Genzyme (Cambridge, MA). Recombinant mouse IL-6 and sIL-6R were prepared from CHO cells transfected with a mouse IL-6 complementary DNA (cDNA) expression vector and a mouse sIL-6R cDNA expression vector, respectively, as reported (4). Purified human collagenase (MMP-1) and gelatinase (MMP-2) were purchased from Yagai Co. Ltd. (Yamagata, Japan). A hydroxamate inhibitor of MMPs [HONHCOCH2CH(i-Bu)CO-Trp-NHMe; GM6001X] was kindly provided by Dr. J. Oleksyszyn (Osteo-Arthritis Science, Inc. Cambridge, MA). All other chemicals were of analytical grade.

Mouse calvarial culture

Five-day-old mice were killed and their calvariae were aseptically harvested and dissected free of suture tissues. The calvariae were divided into paired halves and cultured for 24 h at 37 C under 5% CO_2 in air in 0.5 ml BGJb medium (Gibco BRL, Rockville, MD) containing 1 mg/ml BSA (fraction V, Sigma, St. Louis, MO). After preculture for 24 h, each half calvaria was transferred to fresh medium, with and without respective cytokines, and cultured for an additional 5 days. To determine bone-resorbing activity of test materials, the concentration of calcium in the conditioned medium was measured on day 5 using a calcium kit (Calcium C-test Wako; Wako Pure Chemical, Osaka, Japan). On day 5, to detect osteoclasts, calvariae were fixed with 10% formalin and stained with tartrate-resistant acid phosphatase (TRAP). TRAP-stained calvariae were counterstained with alkaline phosphatase.

Culture of primary mouse osteoblastic cells

Primary osteoblastic cells were isolated from 1-day-old mouse calvariae after five routine sequential digestions with 0.1% collagenase (Wako) and 0.2% dispase (Godo Shusei, Tokyo, Japan), as described (25). Osteoblasts isolated from fractions 3–5 were combined and cultured in α-modified MEM (αMEM), supplemented with 10% FBS at 37 C in a humidified atmosphere of 5% CO_2 in air. To measure steady-state levels of MMP mRNAs, osteoblastic cells were cultured for 24 h in αMEM with 1% FBS and further cultured for an additional 5 days with cytokines.

Northern blot analysis

Total cellular RNA was extracted from cultured mouse calvariae and osteoblastic cells using the acid guanidium-phenol-chloroform method (25). For Northern blotting, 20 μg total RNA were resolved by electrophoresis in a 1% agarose-formaldehyde gel and transferred onto nylon membranes (Hybond N, Amersham, Arlington Heights, IL), then hybridized with a [^{32}P]-labeled cDNA probe, as reported (25). The signals were densitometrically quantified using an image analyzer (Micro Computer Imaging Device, Fuji Film, Tokyo, Japan). Mouse MMP-13 cDNA probe (26) was amplified by RT-PCR (sense primer: 5′ CTTCTGGTCTTCTGGCACACG 3′, antisense primer: 5′ CCCCACCCCATACATCTGAAA 3′) and cut with *Eco*RI, yielding a 485-bp fragment. A 250-bp fragment of human MMP-2 cDNA (27) was used as a probe, which specifically hybridized with mouse MMP-2 mRNA. A 1500-bp fragment of human MMP-3 cDNA was used as a probe for MMP-3 (28). Mouse MMP-9 cDNA probe (29), a 459-bp fragment, was amplified by PCR (sense primer: 5′ TGTTCAGCAAGGGGCGTGTC 3′, antisense primer: 5′ AAACAGTCCAACAAGAAAGG 3′). Human tissue inhibitor of matrix metalloproteinase (TIMP)-1 cDNA was kindly provided by Dr. M. Naruto (Toray Industries, Inc., Kanagawa, Japan).

Assay of collagenase and gelatinase activities

To measure collagenase and gelatinase activities, conditioned media of calvarial cultures were treated for 4 h with 4-aminophenylmercuric acetate (APMA), which activates pro-MMPs into the respective active forms. Collagenase and gelatinase activities were measured by the degradation of fluorescein isothiocyanate (FITC)-labeled type I and type IV collagen using a type I collagenase activity assay kit and a type IV collagenase activity assay kit, respectively (Yagai Co.). One unit of these activities degrades 1 μg of respective collagen per min at 37 C.

Gelatin zymography

Gelatinase activity in the conditioned medium of calvarial cultures was analyzed by zymography after incubation for 4 h, with or without 10 mM APMA, as reported previously (30). Aliquots (10 μl) were mixed with 5 μl of nonreducing SDS-PAGE sample buffer, then subjected to SDS-PAGE using 10% polyacrylamide gels containing 0.6 mg/ml of gelatin. After electrophoresis, gels were incubated for 1 h in washing buffer consisting of 50 mM Tris-HCl, containing 5 mM $CaCl_2$, 1 μM $ZnCl_2$, and 2.5% Triton X-100 to remove SDS, and then in the same buffer without Triton X-100 at 37 C for 3 h. Gels were stained with 0.1% (wt/vol) Coomassie brilliant blue in 50% (vol/vol) methanol, 10% (vol/vol) acetic acid, and destained in a solution of 30% (vol/vol) methanol and 1% (vol/vol) formic acid. Enzyme activity was detected as a clear zone in a darkly stained background.

SDS-PAGE

SDS-PAGE was performed to detect the degradation of native type I collagen by conditioned medium of calvarial cultures. Conditioned medium was incubated with 10 mM APMA for 4 h, and further incubated for 20 h at 37 C with 2 μg purified bovine type I collagen (Yagai Co.); then the reaction was stopped by adding 10 mM EDTA. As standard collagenase and gelatinase, purified human MMP-1 and human MMP-2 (Yagai Co.) were used for incubation with type I collagen. The samples were then subjected to SDS-PAGE using a 10% polyacrylamide gel. After

electrophoresis, the gels were stained with Coomassie brilliant blue solution.

Statistical analysis

Statistical analysis was carried out by Dunnett's *t* test, and the data are expressed as means ± SEM.

Results

Bone-resorbing activities of IL-1 and IL-6

IL-1 markedly stimulates osteoclastic bone resorption *in vitro* by enhancing both osteoclast formation and function. We have reported previously that IL-6 induces osteoclast formation in cocultures of mouse bone marrow cells and osteoblastic cells when sIL-6R is present (4). To compare the bone-resorbing activities of IL-1 and IL-6, we measured the concentration of calcium in the medium on day 5 in mouse calvarial cultures. Both IL-1α (2 ng/ml) and IL-6 (100 ng/ml) in the presence of sIL-6R (200 ng/ml) stimulated bone resorption, but the activity of IL-1α was significantly more potent than that of IL-6 (Fig. 1A). The concentrations of IL-1α, IL-6, and sIL-6R used were the doses sufficient to induce maximal bone resorption. In the cultures treated with 100 ng/ml IL-6, a higher concentration of sIL-6R (400 ng/ml) showed an effect on bone-resorbing activity similar to that induced by 200 ng/ml sIL-6R (data not shown). A large number of TRAP-positive osteoclasts were detected in calvarial tissues cultured with IL-1α or IL-6 with sIL-6R (Fig. 1B). This indicated that both IL-1 and IL-6 markedly stimulate osteoclast formation in calvarial cultures. Therefore, the difference of bone-resorbing activity between IL-1 and IL-6 seems to be caused by other processes of bone resorption, such as matrix degradation regulated by MMPs. When a hydroxamate inhibitor of MMPs was added to calvarial cultures, IL-1-induced bone resorption was markedly inhibited on day 5. In addition, IL-6-induced bone resorption also was suppressed by the same inhibitor to a similar level to IL-1 plus hydroxamate. The medium calcium (mg/dl) levels were: control, 6.43 ± 0.04; IL-1α, 14.04 ± 0.45; IL-1α plus 30 μM hydroxamate, 8.08 ± 0.48; IL-6 with sIL-6R, 9.38 ± 0.25; IL-6 with sIL-6R plus 30 μM hydroxamate, 8.06 ± 0.11.

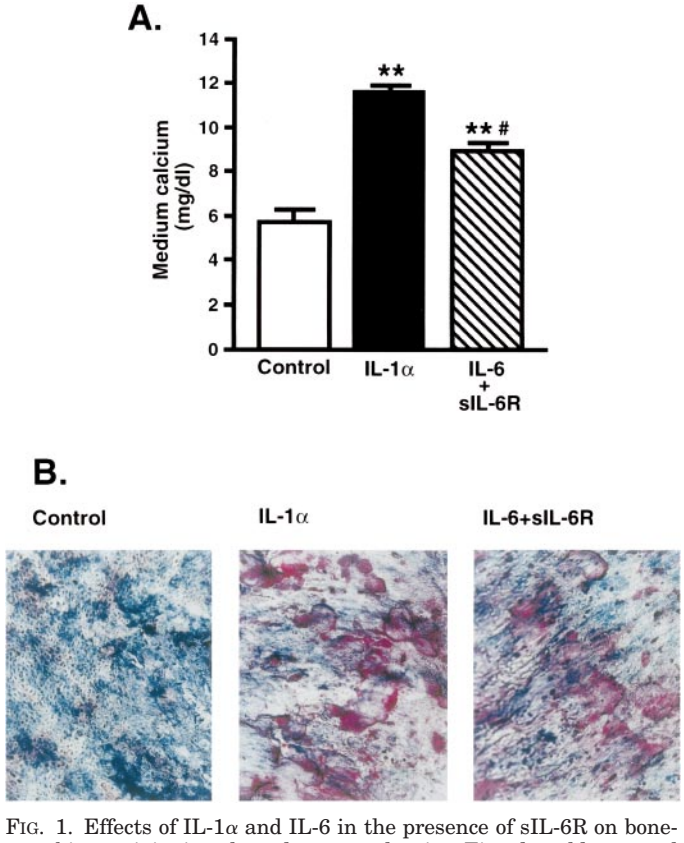

FIG. 1. Effects of IL-1α and IL-6 in the presence of sIL-6R on bone-resorbing activity in cultured mouse calvariae. Five-day-old neonatal mouse calvariae were cultured for 5 days with vehicle, IL-1α (2 ng/ml) or IL-6 (100 ng/ml) with sIL-6R (200 ng/ml). The concentrations of IL-1α, IL-6, and sIL-6R used were the doses sufficient to induce maximal bone resorption. A, Conditioned media were collected, and the concentration of calcium in the medium was measured to monitor the bone-resorbing activity. The data are expressed as means ± SEM of 9 cultures, significantly different from the control (**, $P < 0.01$) and from cultures treated with IL-1α (#, $P < 0.01$). B, Calvariae were fixed and stained with TRAP, shown in *red*, as described in *Materials and Methods*.

Expression of MMP mRNAs in mouse calvariae treated with IL-1 and IL-6 with sIL-6R

To compare the effects of IL-1 and IL-6 on expression of MMP mRNAs, we performed Northern blot analysis using total RNA extracted from mouse calvariae. IL-1α stimulated expression of MMP-13, MMP-2, and MMP-3 mRNAs on day 2, and the enhanced levels were maintained on day 5 (Fig. 2). Treatment with IL-6 together with sIL-6R also stimulated expression of MMP-13, MMP-2, and MMP-3 mRNAs on day 2, but the increased levels decreased to the respective control levels on day 5 (Fig. 2). MMP-3 has been reported to activate other pro-MMPs, which suggests that the induction of MMP-2, -3, and -13 is cooperatively involved in MMP-dependent matrix degradation. Expression of mRNA encoding TIMP-1, a negative regulator of activated MMPs in target tissues, also was enhanced by IL-1 and IL-6 with sIL-6R (Fig. 2). The expression of TIMP-1 mRNA was further increased by IL-1α, but not by IL-6, on day 5 (Fig. 2). Expression of MMP-9 mRNA was similarly enhanced by IL-1 and IL-6 with sIL-6R (Fig. 2).

Collagenase and gelatinase activities

To analyze the functional activities of MMPs expressed in mouse calvariae, collagenase and gelatinase activities in the conditioned medium of calvarial cultures were measured by the degradation of FITC-labeled type I and type IV collagen. IL-1α markedly stimulated both collagenase and gelatinase activities (Fig. 3). In the presence of sIL-6R, IL-6 also stimulated collagenase and gelatinase activities, but the effects were much less than those of IL-1α. The differences between collagenase and gelatinase activities induced by IL-1 and those induced by IL-6 were consistent with the potency of the respective cytokines in inducing expression of MMP-13 and MMP-2 mRNAs (Fig. 2).

Effects of IL-1 and IL-6 on the production of pro- and active-forms of MMP-2

To compare the effects of IL-1 and IL-6 on the production of gelatinases, conditioned media from calvarial cultures

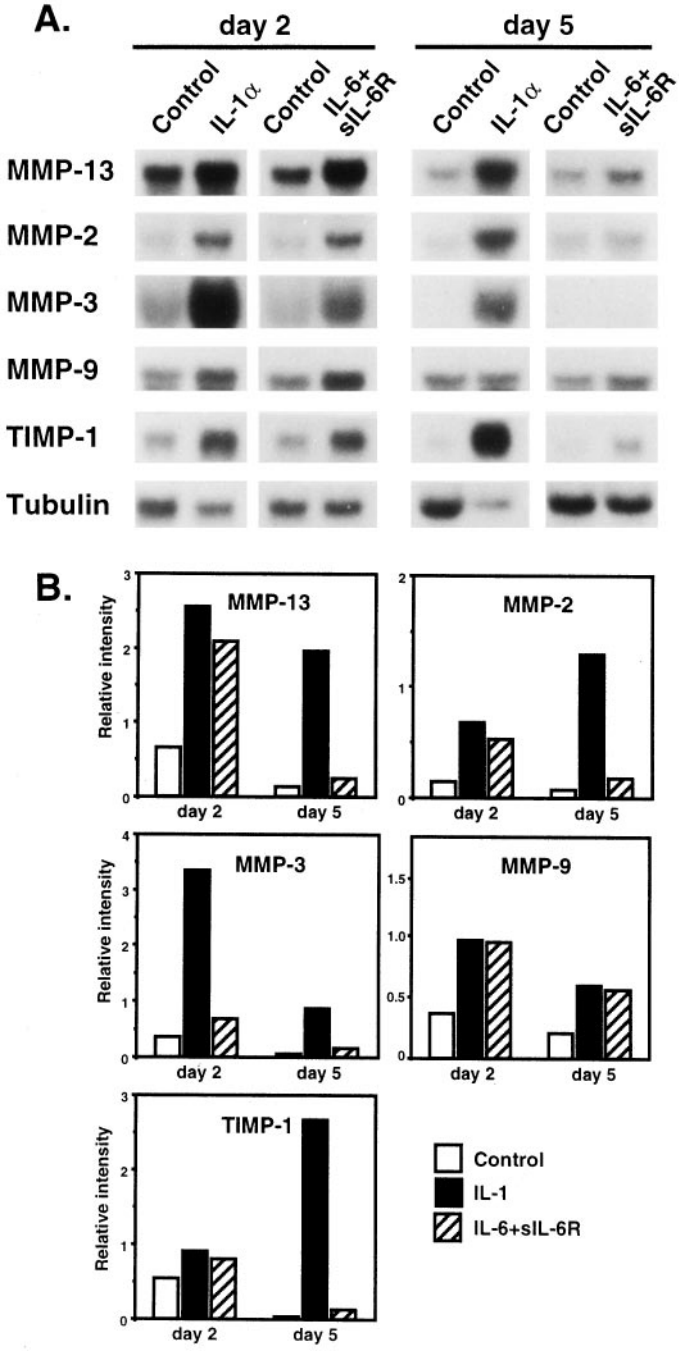

FIG. 2. Effects of IL-1α and IL-6 in the presence of sIL-6R on expression of MMP and TIMP-1 mRNAs in cultured mouse calvariae. A, Mouse calvariae were cultured for 2 or 5 days with IL-1α (2 ng/ml) or IL-6 (100 ng/ml) with sIL-6R (200 ng/ml). Total RNA was extracted, and Northern blotting was performed using [^{32}P]-labeled cDNA probes for MMP-13, MMP-2, MMP-3, MMP-9, TIMP-1, and tubulin, as described in *Materials and Methods*. B, Signals in Northern blots shown in A were quantified and normalized by the expression of tubulin mRNA using an image analyzer.

collected on day 5 were incubated for 4 h, with or without APMA, and used for gelatin zymography. In the absence of APMA, not only pro-MMP-2 (72 kDa) but also a small amount of active-MMP-2 (67 kDa) was detected on the gel from the control cultures. Levels of expression of both pro-

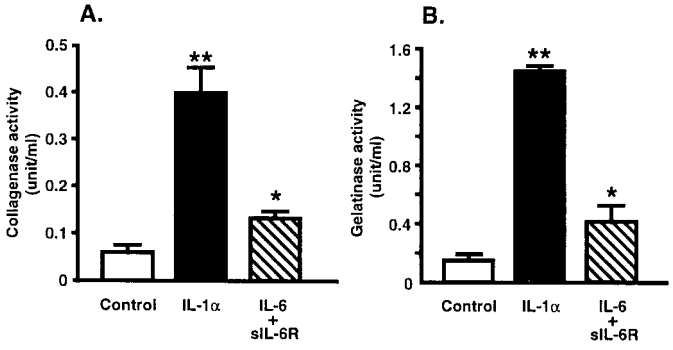

FIG. 3. Effects of IL-1α and IL-6 in the presence of sIL-6R on collagenase and gelatinase activities in the conditioned media collected from cultured mouse calvariae. Mouse calvariae were cultured for 5 days with IL-1α (2 ng/ml) or IL-6 (100 ng/ml) with sIL-6R (200 ng/ml). Conditioned media were collected and treated for 4 h with 10 mM APMA to activate pro-MMPs, then incubated for 4 h with FITC-labeled type I collagen to detect collagenase activity (A) or FITC-labeled type IV collagen for gelatinase activity (B). Both activities were calculated by the degradation of FITC-labeled collagens, as described in *Materials and Methods*. The data are expressed as means ± SEM of 4–6 independent experiments, significantly different from the control (*, $P < 0.05$; **, $P < 0.01$).

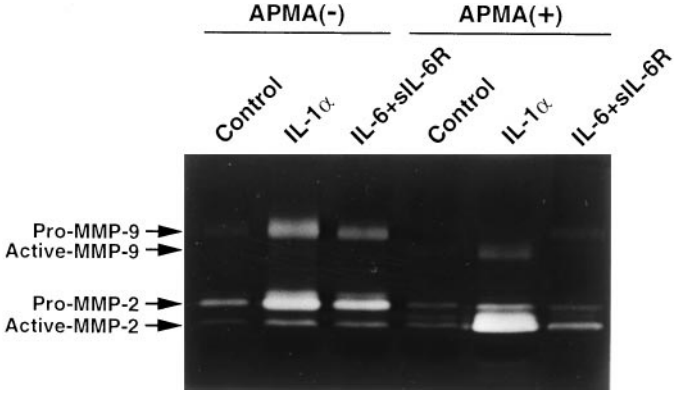

FIG. 4. Detection of MMP-2 and MMP-9 in the conditioned media collected from cultured mouse calvariae, by gelatin zymography. Mouse calvariae were cultured for 5 days with IL-1α (2 ng/ml) or IL-6 (100 ng/ml) with sIL-6R (200 ng/ml), and conditioned media were collected and treated for 4 h with or without 10 mM APMA. For gelatin zymography, the samples were run on a polyacrylamide gel (10%) containing 0.6 mg/ml of gelatin and were analyzed as described in *Materials and Methods*. Gelatinase activities corresponding to pro-MMP-2, active-MMP-2, pro-MMP-9, and active-MMP-9 are indicated by *arrows*.

and active-forms of MMP-2 were markedly enhanced by treatment with IL-1α. Pro-MMP-9 also was detected, and it was markedly enhanced by IL-1. Treatment with IL-6 together with sIL-6R slightly enhanced the production of pro-MMP-2 and pro-MMP-9, but the effect was much less than that of IL-1. When the respective conditioned medium was incubated with APMA, most of the pro-MMP-2 and pro-MMP-9 induced by IL-1α was processed into the respective active forms (Fig. 4). In the presence of APMA, pro-MMP-2 induced by IL-6 was also processed into active-MMP-2, but pro-MMP-9 was not (Fig. 4). The lack of pro-MMP-9 activation by APMA is most likely caused by the short period of time exposed to APMA, as human pro-MMP-9 requires

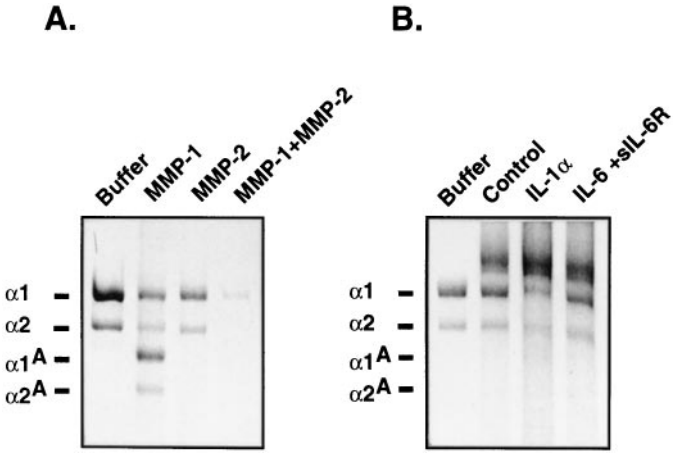

FIG. 5. Effects of the conditioned media collected from cultured mouse calvariae on the degradation of type I collagen. Mouse calvariae were cultured for 5 days with IL-1α (2 ng/ml) or IL-6 (100 ng/ml) with sIL-6R (200 ng/ml). Conditioned media were collected and treated for 4 h with 10 mM APMA to activate pro-MMPs. A, Type I collagen was incubated with purified collagenase (human MMP-1) and/or purified gelatinase (human MMP-2) at 37 C for 20 h. B, Type I collagen was incubated with vehicle or activated conditioned media (control, IL-1α, IL-6 + sIL-6R) at 37 C for 20 h. The generated products were subjected to SDS-PAGE and were stained as described in *Materials and Methods*.

about 48 h incubation at 37 C with APMA to be fully activated (31).

Collagenase and gelatinase cooperatively degrade type I collagen

It is well known that collagenase cleaves native type I collagen into ¾- and ¼-length collagen fragments. Recent studies have indicated that MMP-2 also cleaves native type I collagen under appropriate conditions (24). To analyze collagenolytic activities of conditioned media from calvarial cultures, we performed SDS-PAGE to detect the degraded products of native type I collagen. When type I collagen was incubated with conditioned medium from calvarial cultures treated with IL-1α, levels of both α1 and α2 chains were markedly decreased, but no ¾- or ¼-length fragments, such as $\alpha 1^A$ and $\alpha 2^A$, could be detected (Fig. 5B). Purified collagenase, human MMP-1, cleaved type I collagen to generate α^A fragments, but human MMP-2 did not (Fig. 5A). When type I collagen was incubated with both purified MMP-1 and MMP-2, type I collagen was degraded into smaller fragments but not into α^A fragments (Fig. 5A). The conditioned media from calvarial cultures treated with IL-6 and sIL-6R slightly degraded type I collagen, but the collagenolytic activity was much weaker than that of IL-1α.

Expression of MMP mRNAs in osteoblasts

To examine the regulation of MMPs in bone, we performed Northern blot analysis using osteoblastic cells collected from newborn mouse calvariae. Osteoblastic cells expressed MMP-13 and MMP-2 mRNAs, and the levels of expression of these MMPs were markedly enhanced by IL-1α on days 2 and 5 (Fig. 6). MMP-3 was slightly detected in the control osteoblastic cells, and it was markedly enhanced by treatment with IL-1 on days 2 and 5. IL-6 with sIL-6R similarly induced expression of MMP-2 and MMP-13 mRNAs on day 2, but the stimulated expression of these MMPs was decreased on day 5. MMP-3 mRNA was slightly enhanced by

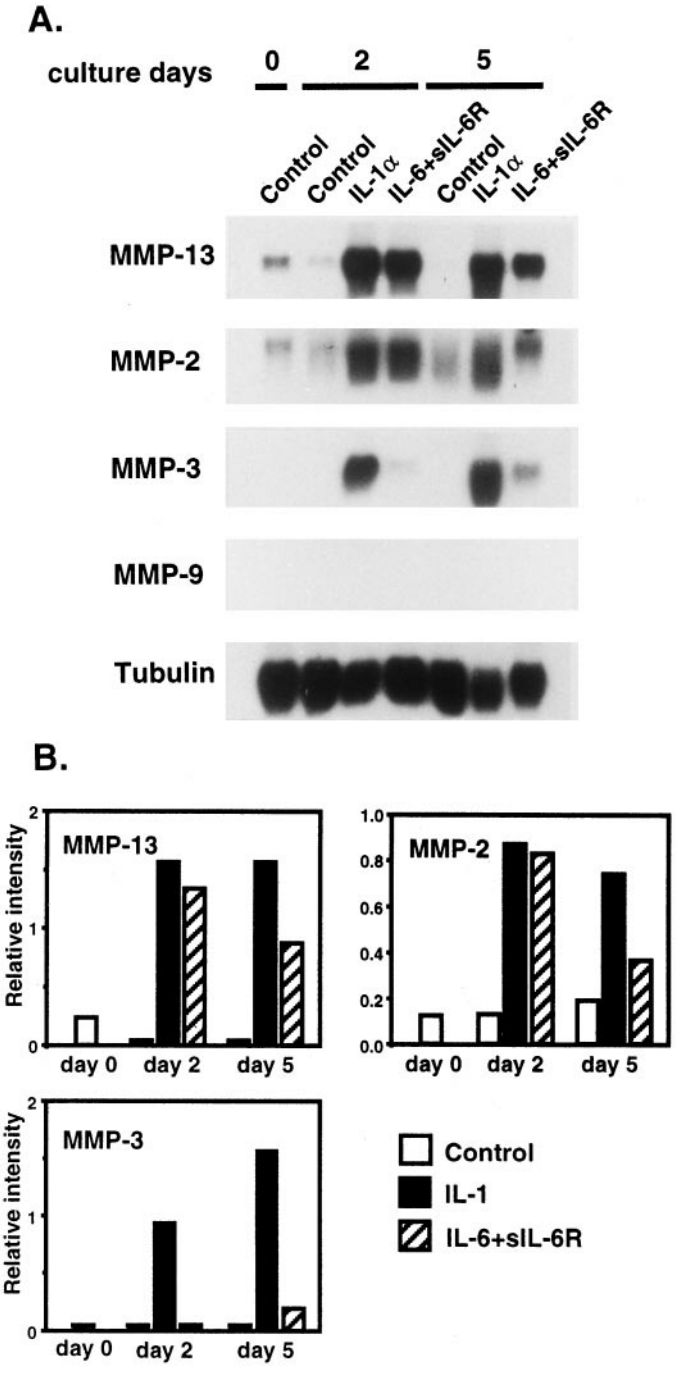

FIG. 6. Effects of IL-1α and IL-6 in the presence of sIL-6R on mRNA expression of MMPs in mouse primary osteoblastic cells. A, Osteoblastic cells were cultured for 24 h in αMEM containing 1% FBS and were further cultured for 2 or 5 days with IL-1α (2 ng/ml) or IL-6 (100 ng/ml) with sIL-6R (200 ng/ml). Total RNA was extracted, and Northern blotting was performed using [^{32}P]-labeled cDNA probes for MMP-13, MMP-2, MMP-3, MMP-9, and tubulin. B, Signals in Northern blots shown in A were quantified and normalized by the expression of tubulin mRNA using an image analyzer.

IL-6 with sIL-6R on day 5. MMP-9 mRNA was not detected in osteoblastic cells, irrespective of the presence or absence of IL-1 and IL-6 (Fig. 6), but it was detected in osteoclast-like cells formed in cocultures of bone marrow cells and osteoblastic cells (data not shown). These results indicate that MMP-2, MMP-3, and MMP-13 are expressed in osteoblasts, and they are markedly enhanced by IL-1 and moderately enhanced by IL-6 with sIL-6R.

Discussion

The present study clearly demonstrated that both IL-1 and IL-6 with sIL-6R induce mRNA expression of various MMPs, including MMP-13, MMP-2, MMP-9, and MMP-3 in mouse calvarial cultures. The potency of IL-1 was much greater and lasted longer than that of IL-6, which was correlated well with the potencies of bone-resorbing activity of these cytokines. We have reported that IL-1 and IL-6 with sIL-6R similarly stimulate osteoclast formation in cocultures of mouse bone marrow cells and osteoblastic cells (4, 6). Consistently, IL-1 and IL-6 with sIL-6R similarly induced osteoclast formation in mouse calvarial cultures (Fig. 1B). In addition to osteoclast formation, matrix degradation is another important step in the process of bone resorption in calvarial cultures. We found that not only MMP-13 (collagenase), but also MMP-2 (gelatinase) and MMP-3 (stromelysin), were markedly induced by IL-1 in calvarial cultures (Fig. 2). In the presence of sIL-6R, IL-6 also stimulated expression of these MMP mRNAs in calvarial cultures but only slightly and rather transiently. As reported previously, IL-1 markedly induces PGE2 production in calvarial cultures (2). The potency of IL-6 in inducing PGE2 synthesis was less than that of IL-1 in calvarial cultures (data not shown). When indomethacin was added to the calvarial cultures, IL-1-induced bone-resorbing activity, as well as collagenase and gelatinase activities, were similarly suppressed to the respective levels induced by IL-6 with sIL-6R (Miyaura, C. *et al*., unpublished results). In addition, PGE2 markedly stimulated the expression of MMP-13 and MMP-2 mRNAs in osteoblasts (data not shown). These findings suggest that PGE2 production is involved in the MMP induction by IL-1 in calvarial cultures.

Transcriptional regulation of collagenases, human MMP-1, and mouse MMP-13 has been reported in various cell types such as connective tissue cells, monocyte-macrophages, and endothelial cells. Human MMP-1 can be stimulated by various growth factors and cytokines, including basic fibroblast growth factor, epidermal growth factor, IL-1, and tumor necrosis factor α. The promoter regions of the genes encoding human MMP-1 and MMP-3 have been sequenced and analyzed. These promoters contain AP-1 sites, and their expression is up-regulated by 12-O-tetradecanoylphorbol-13-acetate and IL-1 (32–34). In contrast, no AP-1 sites have been found in the promoter region of the human MMP-2 gene (35). Neither 12-O-tetradecanoylphorbol-13-acetate nor IL-1 induced MMP-2 mRNA in most cell types reported, except for glomerular mesangial cells (13, 27, 36). In the present study, both IL-1 and IL-6 with sIL-6R markedly induced not only MMP-13 but also MMP-2 mRNA in osteoblasts. Little is known about the effects of bone-resorbing factors on the regulation of MMP-2. Lorenzo *et al.* (16) reported that MMP-2 expressed in osteoblasts was not regulated by bone-resorbing factors. Recently, Franchimont *et al.* (37) reported that IL-6 with sIL-6R caused a marked induction of MMP-13 expression in rat osteoblasts by transcriptional mechanism. Further studies are necessary to examine the mechanism of transcription of MMP-2 and MMP-13 genes by IL-1 and IL-6 in mouse osteoblasts.

MMP-9 has been reported to be localized in monocyte-macrophages and osteoclasts. In the present study, MMP-9 mRNA was not detected in osteoblastic cells (Fig. 6), but it was detected in osteoclasts, using an *in situ* hybridization technique, in calvarial cultures (data not shown). These results are consistent with the previous findings (19, 20, 38). In calvarial cultures, both IL-1 and IL-6 with sIL-6R stimulated the expression of MMP-9 mRNA, which was correlated with the induction of osteoclast-like cell formation (Figs. 1 and 2). Therefore, the increased expression of MMP-9 mRNA in calvarial organ cultures seems to be caused by the increased number of osteoclasts. Jimi *et al*. (39) have reported that IL-1, but not IL-6, directly acts on osteoclast-like cells and supports their survival *in vitro*. However, it is not known whether IL-1 directly regulates MMP-9 expression in osteoclasts. Further studies are needed to define the regulation and biological roles of MMPs in osteoclasts.

The activation of pro-MMPs is essential for matrix degradation. MMP-3 was reported to activate pro-MMPs such as pro-MMP-1 and pro-MMP-9 (40–42). In calvarial cultures, IL-1 markedly induced the expression of MMP-3 mRNA (Fig. 2), whereas IL-6 did so only weakly, even in the presence of sIL-6R. These results are consistent with the observation that both pro- and active-forms of MMP-2 and MMP-9 could be detected in gelatin zymography using conditioned media collected from IL-1-treated cultures (Fig. 4), suggesting that MMP-3 may act as an activator for other pro-MMPs induced by IL-1 and IL-6. Recently, Kinoh *et al.* (43) reported that membrane type (MT)1-MMP was coexpressed with pro-MMP-2 in mouse embryonic osteoblasts. Sato *et al.* (44) also demonstrated the presence of MT1-MMP in rabbit osteoclasts. MT1-MMP was reported to activate pro-MMP-2 and pro-MMP-13 and act as a collagenase (45, 46). Further studies are needed to define the role(s) of MT1-MMP in bone resorption.

Recently, Hill *et al.* (21, 22) reported that synthetic inhibitors of MMPs prevent bone resorption induced by IL-1, 1α,25-dihydroxyvitamin D3, and PTH. Using concentration-dependent selective inhibitors of collagenase and gelatinase, they concluded that both collagenase and gelatinase are involved in bone resorption (22). In the present study, conditioned media from calvarial cultures treated with IL-1 showed marked collagenolytic activity, but the collagenase-induced typical cleavage products of type I collagen, ¾- and ¼-length fragments, could not be detected (Fig. 5B). The degraded pattern of type I collagen was similar to that by simultaneous treatment with purified collagenase and gelatinase (Fig. 5A). Therefore, it is likely that gelatinases are also involved in the subsequent degradation of the collagen fragments cleaved by collagenase in bone.

It is essential to elucidate the distribution of MMPs in bone tissues to determine the selective role of each MMP in bone remodeling. It has been reported that MMP-13 is expressed

preferentially in osteoblasts (8, 10, 17, 18), whereas MMP-9 is expressed selectively in osteoclasts (19, 20, 38). Gack *et al.* (47) demonstrated strong expression of MMP-13 in osteoblastic cells located adjacent to mature osteoclasts. Fuller and Chambers (48) have also reported that MMP-13 mRNA is expressed in osteoblastic cells adjacent to osteoclasts at the sites of active bone resorption. Therefore, MMP-13 in osteoblasts and MMP-9 in osteoclasts may act in concert to promote bone matrix degradation. MMP-13 produced by osteoblasts is responsible for removing the unmineralized osteoid tissues, which protect bone mineral from osteoclastic bone resorption, because osteoclasts cannot adhere to the unmineralized osteoid layer.

In conclusion, IL-1 markedly stimulates expression of MMP-2, -3, and -13 mRNAs in mouse calvariae, but IL-6 stimulates their expression only slightly, even in the presence of sIL-6R. IL-1 and IL-6 similarly induce osteoclast formation, resulting in an increase in the expression of MMP-9 mRNA. Because the differences in the potency of MMP induction between IL-1 and IL-6 correlated well with the bone-resorbing activities of these cytokines, it is likely that the MMP-dependent matrix degradation is the rate-limiting step in osteoclastic bone resorption.

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