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Research Article

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Monocyte Chemoattractant Protein-1 (MCP-1) Expression in Human Articular Cartilage

Induction by Peptide Regulatory Factors and Differential Effects of Dexamethasone and Retinoic Acid

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Abstract

Monocyte influx and activation in synovial joints are important in the pathogenesis of both degenerative and inflammatory arthropathies. In this study, we demonstrate the potential of articular cartilage to directly modulate these events.

IL-1-stimulated human articular chondrocytes transcribed 0.7-kb monocyte chemoattractant protein-1 (MCP-1) mRNA. In situ hybridization of cartilage organ cultures revealed MCP-1 transcripts in chondrocytes in the superficial tangential zone within 2 h of stimulation with IL-1. Chondrocytes in deeper layers responded by 4 h and reached maximum MCP-1 mRNA levels by 8–12 h. IL-1-stimulated cartilage organ and chondrocyte monolayer cultures released functional monocyte chemotactic activity. This was neutralized by a monoclonal antibody specific for MCP-1, and was associated with the synthesis and secretion of immunoreactive 13-kD and 15-kD isoforms of MCP-1.

Regulators and signal transduction pathways involved with the expression of the MCP-1 gene in chondrocytes were analyzed. Steady-state mRNA levels were increased by the known chondrocyte activators IL-1, tumor necrosis factor alpha, LPS, platelet-derived growth factor, and transforming growth factor beta. In addition, leukemia inhibitory factor induced MCP-1 gene expression and protein synthesis, identifying this cytokine as a new regulator of chondrocyte function. Dexamethasone blunted the induction of MCP-1 gene expression by IL-1 and by activators of protein kinase A as well as protein kinase C signal transduction pathways. In contrast, retinoic acid strongly increased phorbol myristate acetate-induced MCP-1 expression and potentiated the effects of IL-1 and LPS.

In conclusion, chondrocytes express MCP-1 in response to factors that are present in cartilage or synovium. This provides a mechanism by which cartilage can play an active role in the initiation and progression of arthritis. (*J. Clin. Invest.* 1992; 90:488–496.) Key words: chondrocytes • cartilage • monocyte chemoattractant protein-1 • leukemia inhibitory factor • transforming growth factor beta • retinoic acid

Introduction

Arthritis is generally viewed as a process that is based on the infiltration of synovial tissue and/or fluid with inflammatory cells that cause acute symptoms and lead to degradation of cartilage. In this scenario, cartilage is seen as the passive target of synovial inflammation, a notion that has been revised by the demonstration that chondrocytes are secretory cells that produce not only proteases (1) but also multiple regulatory factors that can act on cartilage, synovium, or on leukocytes infiltrating the joint (2–6).

Synovial fluid monocytoysis is a finding characteristic of osteoarthritis (7) a disease originating in and primarily affecting cartilage. Furthermore, intraarticular monocyte infiltration and activation are thought to be important events in the pathogenesis of cartilage degradation in inflammatory and degenerative arthropathies. Monocyte recruitment in arthritides may be initiated by a variety of chemotactic factors derived from articular tissues, including type II collagen peptides released by proteolysis of cartilage (8), and transforming growth factor beta (TGF β)¹ and platelet-derived growth factor (PDGF) secreted from synoviocytes and chondrocytes (5, 6). In this study, we examine the ability of articular cartilage to express monocyte chemoattractant protein (MCP-1), a more recently identified cytokine (9–13) that is a member of a family of small, proinflammatory polypeptides that includes platelet factor 4 and IL-8 (14, 15). MCP-1 is specifically recognized by monocytes, stimulating not only chemotaxis but also the release of superoxide and lysosomal proteases (16). MCP-1 is secreted as a 76-amino acid glycoprotein that migrates typically in two molecular forms (13 and 15 kD) on SDS-PAGE under reducing conditions (12). The gene for MCP-1 has been cloned, and some of its regulatory elements have been characterized (17).

Although multiple inducers and cell sources of MCP-1 have been characterized, expression by joint tissue cells is largely unknown. This study demonstrates that chondrocytes express the MCP-1 gene and that MCP-1 is the major monocyte chemotactant in IL-1-stimulated cartilage organ culture. Regulatory factors that are known to be present in cartilage or synovium can induce MCP-1 gene expression by chondrocytes in monolayer and organ culture. New agents and pathways in-

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1. Abbreviations used in this paper: LIF, leukemia inhibitory factor; MCP, monocyte chemoattractant protein; PDGF, platelet-derived growth factor; RA, retinoic acid; TGF β , transforming growth factor beta; TNF α , tumor necrosis factor alpha.

volved with the induction of MCP-1 in chondrocytes are identified.

Methods

Chondrocyte isolation and culture. Cartilage was obtained at autopsy from donors without known history of joint disease. For all experiments reported here, cartilage from the femoral condyles and tibial plateaus of the knee joints was used. Cartilage slices were prepared and washed with DMEM (Whittaker M.A. Bioproducts, Walkersville, MD). They were minced with a scalpel and treated with trypsin (10% vol/vol) for 15 min in a 37°C waterbath. The samples were transferred to DMEM containing 5% fetal bovine serum (FBS), penicillin-streptomycin-fungizone, and 2 mg/ml clostridial collagenase type IV (Sigma Chemical Co., St. Louis, MO) and digested for 3 h on a gyratory shaker until the tissue fragments were dissolved. The cells were washed three times and cultured as primary chondrocytes. For cartilage organ culture experiments, slices of knee cartilage of 0.5 × 0.5 × 0.3-cm size were prepared. Care was taken not to disturb the surface and to obtain full thickness cartilage. Slices were weighed and cultured in 12-well plates in DMEM 1% FBS. For studies on gene expression chondrocytes were used as primary or subcultured cells as indicated for each experiment.

Chemotaxis assay. Enriched preparations of human peripheral blood monocytes were isolated from the heparin-anticoagulated venous blood of normal volunteers. First, mononuclear leukocytes were separated by centrifugation at 400 g for 30 min over Ficoll-Hypaque, washed three times, and resuspended in RPMI 1640 that contained 10% FBS. 3-ml aliquots of mononuclear leukocytes were carefully layered over a discontinuous Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient (5.0 ml 30% Percoll; 5.0 ml 40% Percoll; 15.0 ml 50.5% Percoll; 7.5 ml 75% Percoll) in 50-ml centrifuge tubes. After centrifugation at 400 g for 30 min at 22°C, the monocyte-enriched fraction was removed from the 40–50.5% interface of each gradient. Monocyte enrichment was typically > 85% under these conditions, as assessed by staining with the mature monocyte marker Mo2 (CD14).

The monocyte-enriched fraction was washed three times in RPMI 1640, and resuspended at 10⁶ cells/ml in RPMI 1640, supplemented with 10 mM HEPES, 1% penicillin-streptomycin, and 1% BSA. Monocyte chemotaxis assays were performed in triplicate in a 48-well modified Boyden chamber apparatus (Neuro Probe, Cabin John, MD) (18). Monocytes were separated from purified agonists (2 nM human recombinant MCP-1, purchased from Biosource, Camarillo, CA) or 10 nM FMLP, purchased from Sigma Chemical Co. (St. Louis, MO) or from chondrocyte-conditioned media by a polycarbonate polyvinylpyrrolidone-free filter with a pore size of 5 µm (Neuroprobe). Before testing, chemoattractants (0.1 ml) were allowed to equilibrate (37°C for 30 min, 4°C for 1 h) with (0.01 ml) of buffer, or 38 nM nonimmune mouse IgG, or a previously characterized, neutralizing murine monoclonal antibody to MCP-1 (antibody E11 [19], a generous gift from Drs. Ed Leonard and Teizo Yoshimura, National Cancer Institute, Frederick, MD). Cells (10⁶/ml in 0.05 ml) were incubated with chemoattractants (0.025 ml) for 90 min at 37°C, followed by Wright-Giemsa staining. Cell migration to the underside of the filter was quantified by reading five high power fields for each well.

Metabolic labeling and immunoprecipitation. MCP-1 protein synthesis was analyzed by metabolic labeling and immunoprecipitation. Primary chondrocytes were washed in PBS and incubated in cysteine- and methionine-free RPMI (ICN Biomedicals, Inc., Costa Mesa, CA) supplemented with L-Glu, L-Met, 1% FBS, [³⁵S]cysteine, and [³⁵S]methionine (ICN Biomedicals, Inc.; 100 µCi/ml) for 48 h. The supernatants were collected and precleared by incubating with protein G sepharose (Zymed Lab, Inc., South San Francisco, CA) for 1 h. The samples were incubated overnight in the presence of the monoclonal murine antibody E11 (19) to MCP-1 (2 µg/sample) on a rotor, and

protein G sepharose was added for one additional hour. The precipitates were washed four times with PBS containing 0.05% Tween-80, 0.1% Triton X-100, and 1 mM PMSF followed by two washes in PBS. The beads were boiled for 5 min in Laemmli sample buffer and the proteins were separated on 15% polyacrylamide gels under reducing conditions. After staining with Coomassie blue the gels were treated with Amplify (Amersham Corp., Arlington Heights, IL) for 1 h, dried, and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY) at -70°C for 2 d.

RNA probe preparation. The cDNA for MCP-1 inserted in EcoRI sites of pBS-SK(-) was kindly provided by Drs. Ed Leonard and Teizo Yoshimura, National Cancer Institute, Frederick, MD. For development of a probe for β-actin, two 15-bp oligonucleotides (5'-CGTCGTCGACAACGG-3' and 5'-GACCGTAGCACTACC-3') defining a 216-bp fragment of the cDNA were designed. The restriction sites EcoRI and HindIII were added at their 5' ends, respectively. After amplification by polymerase chain reaction the fragment was inserted into pGEM-4z (Promega Corp., Madison, WI).

The recombinant plasmids were linearized and transcribed with the T7 or T3 RNA polymerase to obtain antisense and sense probes, respectively. For Northern blotting, the probes were labeled with [³²P]UTP (Amersham) and separated from unincorporated nucleotides by gel filtration (Centri-sep columns; Princeton Separations, Inc., Adelphia, NJ). The probes for in situ hybridization were generated in the presence of ³⁵S-UTP. After transcription, the template was digested with DNase and the products were recovered by phenol extraction and ethanol precipitation. The specific activity was ~ 2 × 10⁸ dpm/µg template.

Northern blot hybridization. Primary or subcultured (P1 to P8) chondrocytes were maintained in T175 flasks and stimulated as indicated for each experiment. Total RNA was extracted by the single step guanidinium thiocyanate-phenol-chloroform method (20). 10–30 µg of total RNA was separated on 1% formaldehyde gels, blotted onto nylon filters, and cross-linked with UV light for 5 min each side. The blots were prehybridized in 50% formamide, 6× standard saline citrate (SSC), 0.5% SDS, 0.1% Tween 20, 100 µg yeast RNA/ml for 15 min at 65°C. The prehybridization mixture was replaced with fresh solution containing 10⁶ cpm/ml of probe. Hybridization was performed overnight at 65°C and followed by washes in 1× SSC, 0.1% SDS at room temperature (2 × 30 min) and 0.1× SSC, 0.1% SDS at 65°C (2 × 30 min). The damp filters were exposed to Kodak XAR film at -70°C for 2–24 h. To confirm equal RNA load and complete transfer the 18S and 28S bands were visualized with ethidium bromide on all filters. In addition, RNA load was examined by probing for β-actin mRNA. For most aspects of MCP-1 mRNA expression at least three separate experiments were performed.

In situ hybridization. Cartilage organ cultures were prepared as outlined above. After appropriate incubation time the slices were cut perpendicular to the surface into two pieces of equal size. They were snap frozen in dry ice/2-methyl-butane and stored at -70°C until use. Frozen sections (5 µm) were cut and mounted onto precleaned slides (Fisher Scientific, San Francisco, CA). They were fixed in 4% paraformaldehyde, washed in 1× PBS, and transferred to 2× SSC.

Hybridization was performed as described elsewhere (21). In brief, the specimens were acetylated in triethanolamine with acetic anhydride and incubated in glycine buffer. Hybridization was carried out with 10⁵ cpm/µl of probe in 50% formamide, 3× SSC, 500 µg/ml yeast tRNA, 1 mg/ml single-stranded calf thymus DNA, 2 mg/ml BSA, 10 mM DTT, and 1% polyethylene glycol overnight at 50°C. Posthybridization treatment consisted of washes with 2× SSC, 50% formamide at 50°C, incubation with RNase solution and further washes in 2× SSC at 55°C and thereafter in 2× SSC at room temperature. Specimens were dehydrated, air-dried, and covered with Kodak NTB nuclear track emulsion (Eastman Kodak) for autoradiography. After exposure for 3–6 d at 4°C, the slides were developed, fixed, and counterstained with Giemsa. Cells were considered positive, when there were > 20 granules over nucleus and cytoplasm. A replicate slide was hybridized with sense

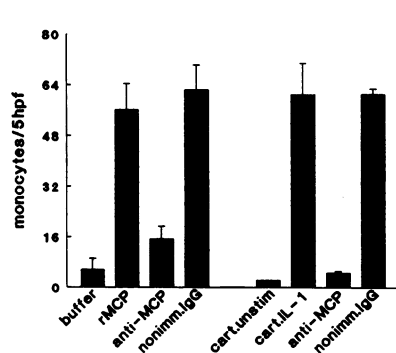


Figure 1. IL-1-stimulated cartilage secretes biologically active MCP-1. Cartilage fragments (from a 37-yr-old donor) were incubated in DMEM + 1% FBS for 24 h with or without IL-1 β (10 ng/ml). Conditioned media were analyzed for monocyte chemotactic activity using a multi-well Boyden chamber

assay as described in Methods. Conditioned media and rMCP-1 (0.1 ml) were pretreated with 0.01 ml buffer, or murine anti-MCP-1 Ab, or nonimmune mouse IgG at a final concentration of 3.8×10^{-8} M. Human rMCP-1 was used as a chemoattractant at a final concentration of 2×10^{-9} M. The results represent mean values of triplicate determinations.

probe or with an unrelated antisense probe to serve as negative controls for each condition.

Reagents. The following reagents were used: recombinant human TGF β 1, PDGF, IL-1 β , tumor necrosis factor alpha (TNF α), leukemia inhibitory factor (LIF), and IL-6 (R&D Systems, Minneapolis, MN); retinoic acid, dexamethasone, phorbol myristate acetate (PMA), cAMP, and LPS from *Salmonella Minnesota* (Sigma Chemical Co.).

Results

IL-1-stimulated cartilage secretes biologically active MCP-1. To assess whether chondrocytes have the capacity to release biologically active MCP-1, conditioned media of cartilage in organ cultures were tested in a human monocyte chemotaxis assay. The conditioned media of IL-1-stimulated, but not unstimulated cartilage, contained monocyte chemoattractant activity comparable to the bioactivity elicited by 2 nM human recombinant MCP-1 (Fig. 1). Conditioned media from 24-h cultures were tested since this is an optimal time point for the induction of cytokine release by IL-1 in chondrocytes. The chemotactic activity of both rMCP-1 and the conditioned media of IL-1-stimulated cartilage, were nearly completely abrogated by pretreatment with a monoclonal, neutralizing antibody to MCP-1, but not with nonimmune murine IgG (Fig. 1). Specificity of this assay system for MCP-1-induced monocyte chemotaxis was validated by: (a) establishing that the human neutrophil and T lymphocyte chemotactant, IL-8 (10 nM) did not induce a chemotactic response (not shown); and (b) the lack of a neutralizing effect of identical pretreatment of 10 nM FMLP with monoclonal anti-MCP-1 antibody on the elicited monocyte chemotaxis response (90 ± 3 monocytes/five high power fields without anti-MCP-1, 110 ± 18 monocytes/five high power fields with anti-MCP-1, $n = 3$). Furthermore, the addition of IL-1 in doses up to 10 ng/ml to culture media did not induce detectable monocyte chemotaxis. Thus, acti-

vated chondrocytes in organ culture secreted biologically active MCP-1, which was the predominant chemoattractant for monocytes in their conditioned media.

Pattern of chondrocyte MCP-1 mRNA expression in response to IL-1 in cartilage organ culture. Many parameters of the responsiveness of chondrocytes within cartilage to stimulation with IL-1 have not previously been characterized. To examine kinetics, the distribution and number of cells that express MCP-1 mRNA, cartilage fragments of identical size and shape containing all layers of the tissue, were cut and cultured in the presence or absence of IL-1 (10 ng/ml). Fig. 2 shows that unstimulated cells (Co) remained negative for MCP-1 mRNA as assayed by in situ hybridization. By 2 h after stimulation with IL-1, the majority of the cells in the superficial tangential zone showed some positivity for MCP-1 mRNA (white granules are covering cells in dark-field picture). However, by 4 h, superficial cells were again negative, while cells in deeper location (round cells in chondrons) showed an increase in the number of positive cells and in the intensity of the hybridization signal per cells. After 6 h it was predominantly chondrocytes in deeper cell layers that were strongly positive (*bottom panel*). Additional experiments showed that cells in deeper location remained positive for at least 18 h (not shown).

Stimulation with LPS (1 μ g/ml), which readily activates MCP-1 expression in chondrocyte monolayer cultures (see results below) resulted only in a weak response of cells in superficial location. Only in the presence of high concentrations of LPS ($> 10 \mu$ g/ml) some cells in deeper layers responded. Collectively, these results demonstrate that chondrocytes when surrounded by extracellular matrix in cartilage organ culture respond to stimulation with IL-1 with the expression of MCP-1 mRNA. The response is rapid, and the IL-1 effect is propagated through cartilage matrix to chondrocytes in deeper locations. In contrast, LPS penetration through the matrix appears to be poor, and was associated largely with activation of cells in the superficial zone.

Chondrocytes synthesize and release the 13- and 15-kD isoforms of MCP-1. IL-1-activated cartilage expressed MCP-1 mRNA and secreted biologically active MCP-1. We next analyzed whether activated chondrocytes synthesize this cytokine and characterized the MCP-1 proteins produced. Primary chondrocytes were cultured in the presence of [35 S]methionine and [35 S]cysteine for 48 h, and stimulated with IL-1 (10 ng/ml) or LIF (10 ng/ml). Conditioned media were immunoprecipitated, and analyzed by a 15% SDS-PAGE under reducing conditions (Fig. 3). Chondrocytes synthesized two isoforms of MCP-1 in similar quantity, and of the characteristic 13-kD and 15-kD sizes (12). Stimulation with not only IL-1, but also with LIF induced a marked increase in the intensity of the bands, demonstrating upregulation of protein synthesis in response to these cytokines.

Regulation of MCP-1 gene expression in chondrocytes. We further evaluated regulation of MCP-1 expression using cultured chondrocytes. Northern blot analysis showed that pri-

Figure 2. Time- and site-specific expression of MCP-1 mRNA by chondrocytes in cartilage organ culture. Cartilage fragments were cultured in DMEM + 1% FBS in the absence (Co) or presence of IL-1 (10 ng/ml) for 2 h, 4 h, and 6 h. MCP-1 mRNA expression was analyzed by in situ hybridization (positive cells are covered with white granules in dark-field pictures). Unstimulated chondrocytes were negative (Co). After 2-h stimulation positive cells were mainly located in the superficial tangential zone. After 4 h and 6 h superficial cells became negative, but abundant MCP-1 mRNA was then found in cells in the deeper layers. Each section was photographed in bright field (*left panels*) or dark field (*right panels*).

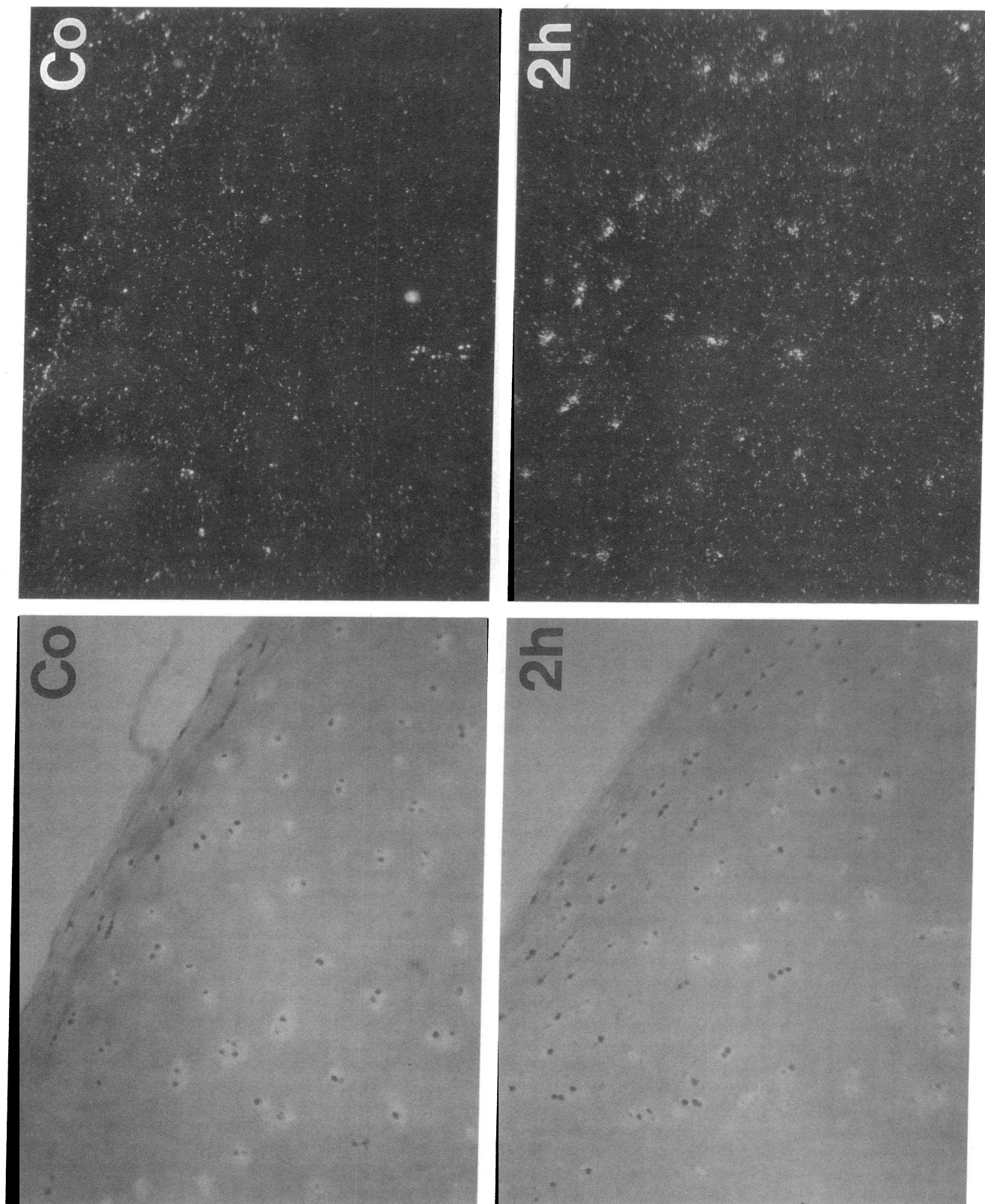


Figure 2

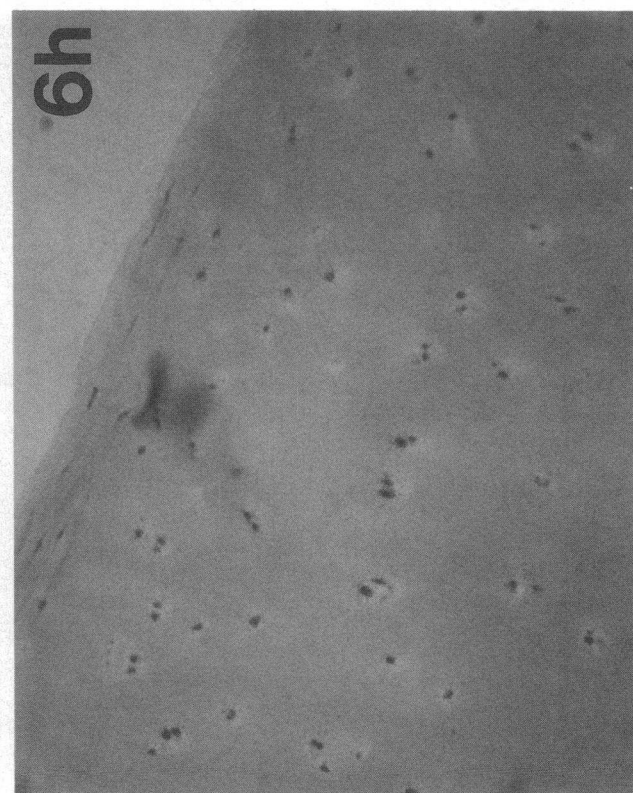
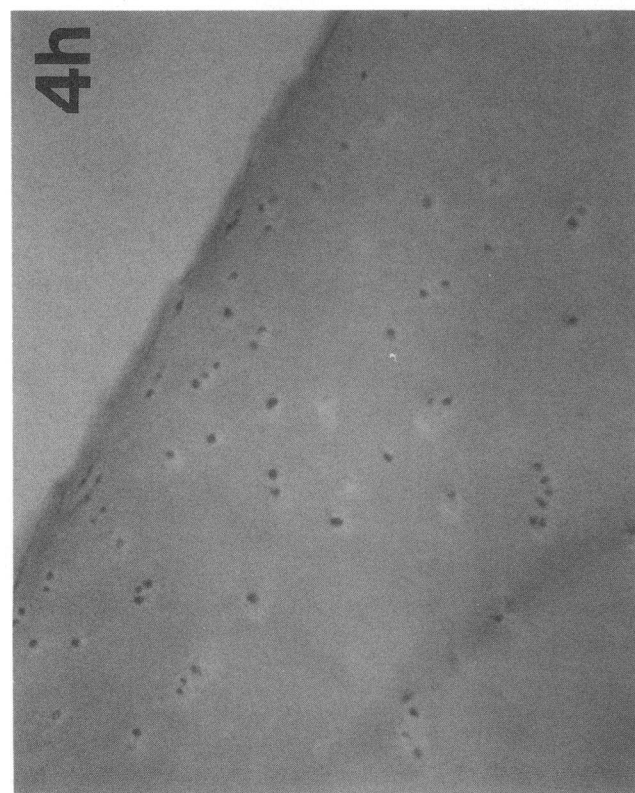
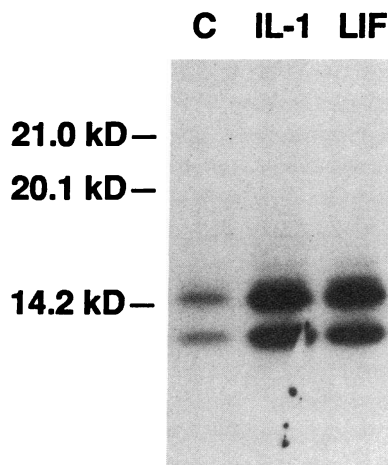


Figure 2 (Continued)



LIF (both stimuli at a concentration of 10 ng/ml). MCP-1 proteins appear as the characteristic doublet at 13 kD and 15 kD.

Figure 3. Chondrocytes secrete de novo synthesized MCP-1 in response to stimulation with IL-1 or LIF. Primary chondrocytes were cultured for 48 h in the presence of [35 S]-methionine and [35 S]-cysteine. Conditioned media were immunoprecipitated and analyzed by SDS-PAGE under reducing conditions. Lane 1 shows unstimulated cells, lane 2 stimulation with IL-1, lane 3 stimulation with

many chondrocytes cultured in the presence of serum consistently expressed 0.7-kb MCP-1 mRNA (see results below). This decreased to low levels after overnight serum-starvation (Fig. 4, lane 1) and remained low over a period of at least 48 h (not shown). IL-1 β , TNF α , and LPS (lanes 2, 5, and 6, respectively) induced a marked increase in MCP-1 mRNA levels while IL-6, an important regulator of chondrocyte function (22, 23) had no detectable effect (lane 3). Surprisingly, LIF caused only a slight increase in MCP-1 mRNA level at 5 h (lane 4). This was not expected because of the marked effect of LIF on MCP-1 synthesis (Fig. 3) and suggested that the kinetics of MCP-1 expression in response to LIF differed from the kinetics in response to IL-1.

We thus compared the kinetics of MCP-1 mRNA expression in response to IL-1, LIF, and TGF β (Fig. 5), and observed that LIF induced a very rapid increase in MCP-1 mRNA, which reached its maximal levels by 2 h. By 5 h, the mRNA levels had already returned to baseline (Fig. 5 B). As we recently showed that LIF increases the expression of LIF mRNA (24) the same blot that had been probed for MCP-1 was hybridized to a labeled LIF RNA probe. This showed that in contrast to MCP-1, LIF mRNA levels remain elevated for at least 9 h. The very rapid and transient induction of high MCP-1 mRNA levels by LIF was confirmed in two additional experiments.

TGF β was identified as an additional new inducer of MCP-

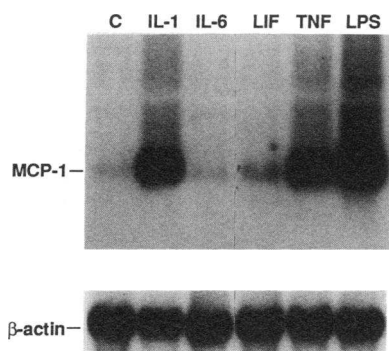


Figure 4. Chondrocytes express MCP-1 mRNA. Primary chondrocytes (from a 31-yr-old donor) were stimulated for 5 h with IL-1 (10 ng/ml; lane 2), IL-6 (10 ng/ml; lane 3), LIF (10 ng/ml; lane 4), TNF α , (10 4 U/ml; lane 5), LPS (1 μ g/ml, lane 6); unstimulated cells in lane 1. Total RNA was extracted and subjected

to Northern blotting. After hybridization with an MCP-1 RNA-probe the filter was hybridized to a β -actin probe to assess the amount of RNA per lane.

1 mRNA expression. Its effects occurred more slowly with a maximum after 5–8 h (Fig. 5 C). MCP-1 mRNA levels often remained elevated for more than 11 h in response to TGF β and were only slightly lower than those seen after stimulation with IL-1. PDGF induced MCP-1 gene expression with kinetics comparable to those observed with IL-1 (not shown). Collectively, these experiments identify LIF and TGF β as two new inducers of MCP-1. The distinct patterns of MCP-1 mRNA induction suggest that IL-1, LIF, and TGF β may use different mechanisms to regulate MCP-1 levels.

Dexamethasone and retinoic acid differentially affect MCP-1 gene expression. Glucocorticoids such as dexamethasone inhibit IL-1 responses in a variety of cells and suppress the expression of IL-1-inducible cytokines (25). Fig. 6 A shows that chondrocyte culture in 1% FBS is sufficient to induce detectable levels of MCP-1 mRNA (lane 1). Dexamethasone (lane 3) abrogated the serum-induced MCP-1 gene expression and markedly reduced the response to IL-1 (lane 4). In addition, dexamethasone inhibited MCP-1 expression in response to cAMP and to PMA (Fig. 6 B). Thus, dexamethasone is a general inhibitor of MCP-1 expression in chondrocytes inhibiting serum-induced (basal) and inducible gene expression.

Retinoic acid (RA) has potent effects on differentiation of mesoderm-derived tissues (26) and inhibits IL-1-induced expression of collagenase and stromelysin and production of proinflammatory cytokines (27–29). Fig. 7 A shows that stimulation of chondrocytes with RA (1 μ M) in serum containing medium for 72 h increased MCP-1 mRNA levels (lane 2). This RA concentration of 1 μ M was optimal for the induction of these effects. Dexamethasone, as documented above, reduced serum-induced MCP-1 expression (lane 3), and this was re-

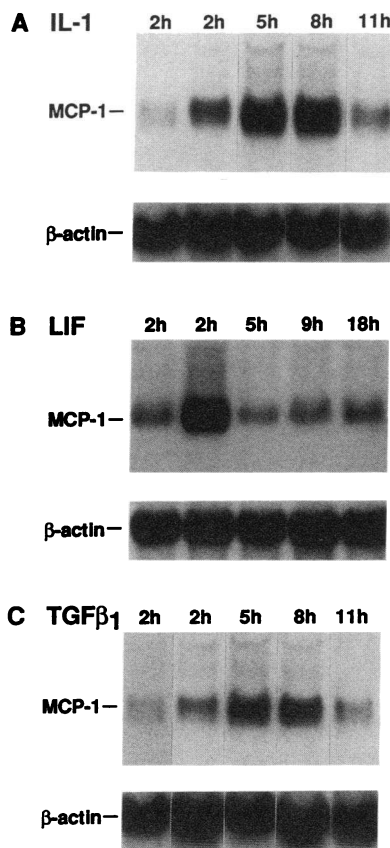


Figure 5. IL-1, LIF, and TGF β induce MCP-1 expression with different time kinetics. Subcultured chondrocytes were serum-starved for 24 h and then stimulated with IL-1 (A), LIF (B), or TGF β (C) (all stimuli at 10 ng/ml) for times indicated above the lanes. First lane in all panels represent unstimulated cells. Total RNA was extracted, subjected to Northern blotting, and analyzed with an MCP-1 RNA probe. To assess the amount of RNA per lane the filter was subsequently hybridized to a β -actin probe.

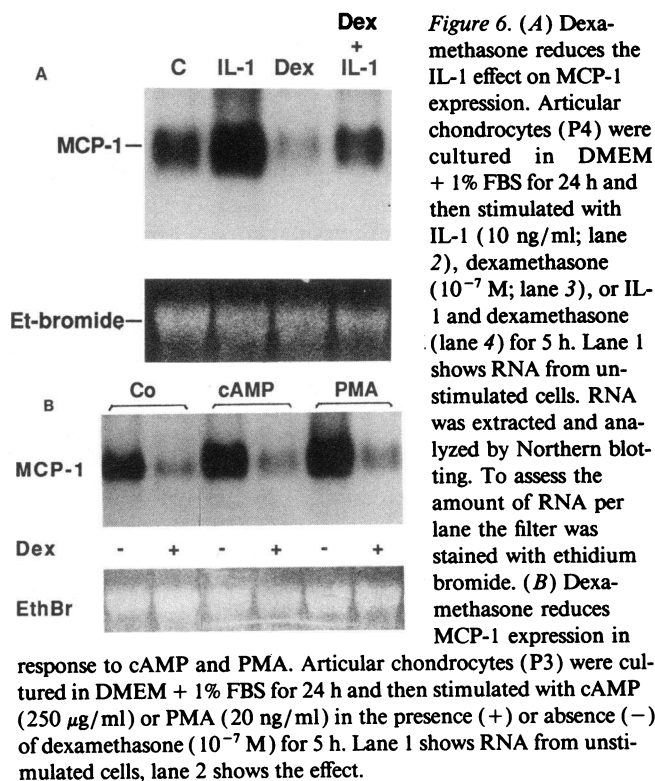


Figure 6. (A) Dexamethasone reduces the IL-1 effect on MCP-1 expression. Articular chondrocytes (P4) were cultured in DMEM + 1% FBS for 24 h and then stimulated with IL-1 (10 ng/ml; lane 2), dexamethasone (10^{-7} M; lane 3), or IL-1 and dexamethasone (lane 4) for 5 h. Lane 1 shows RNA from unstimulated cells. RNA was extracted and analyzed by Northern blotting. To assess the amount of RNA per lane the filter was stained with ethidium bromide. (B) Dexamethasone reduces MCP-1 expression in

response to cAMP and PMA. Articular chondrocytes (P3) were cultured in DMEM + 1% FBS for 24 h and then stimulated with cAMP (250 μ g/ml) or PMA (20 ng/ml) in the presence (+) or absence (-) of dexamethasone (10^{-7} M) for 5 h. Lane 1 shows RNA from unstimulated cells, lane 2 shows the effect.

versed by RA (lane 4). Furthermore, as shown in Fig. 7 B, RA not only elevated serum-induced MCP-1 expression but also markedly upregulated the MCP-1 response to IL-1, LPS, and PMA (Fig. 7 B). To test whether this effect was limited to MCP-1 gene expression or was a more general phenomenon the same Northern blot was sequentially probed for IL-6, IL-8, and LIF mRNAs. With all stimuli that induce expression of these cytokines a marked upregulation by RA was observed. Thus, in contrast to its effect on cytokine expression in monocytes (29), RA is a potent stimulus of MCP-1 gene expression in chondrocytes and can upregulate the effect of different agents that utilize distinct mechanisms in the induction of MCP-1.

Discussion

This study shows the expression of biologically active MCP-1 by chondrocytes in human articular cartilage. We have characterized the kinetics and distribution of cells expressing MCP-1 in cartilage organ culture by in situ hybridization and have carried out a detailed analysis of inducers, inhibitors, and signal transduction pathways, identifying LIF, TGF β , and RA as new activators of MCP-1 expression.

MCP-1 is a member of a recently identified family of proinflammatory cytokines (14, 15). Its principal biological functions are monocyte chemotaxis and activation. The findings of this study suggest that, through the secretion of biologically active MCP-1, chondrocytes have the potential to attract monocytes into synovial fluid and membrane, to activate oxidative metabolism and protease release and thus to actively promote cartilage degradation. The production of MCP-1 by chondrocytes also represents an important pathogenetic interaction be-

tween synovium and cartilage. Through the release of MCP-1, mononuclear phagocytes are attracted to cartilage and this may be one factor that directs the proliferation of rheumatoid pannus onto the articular surface. Conversely, the activated proliferating synovium is a source of factors such as IL-1, TNF α , LIF, TGF β , and PDGF that stimulate or perpetuate chondrocyte MCP-1 expression and this can create a circuit that is likely to result in cartilage destruction.

Having established that chondrocytes can produce MCP-1, this study defined characteristics of expression of this gene that are important or unique to cartilage. The first point addressed the role of extracellular matrix in the induction and secretion of MCP-1. We have demonstrated that cells in cartilage organ cultures are not only induced to produce MCP-1, but that biologically active MCP-1 is secreted from the tissue into the culture media. Although MCP-1 is a heparin-binding protein, substantial amounts of this cytokine were released from cartilage, which contains a proteoglycan-rich matrix. The func-

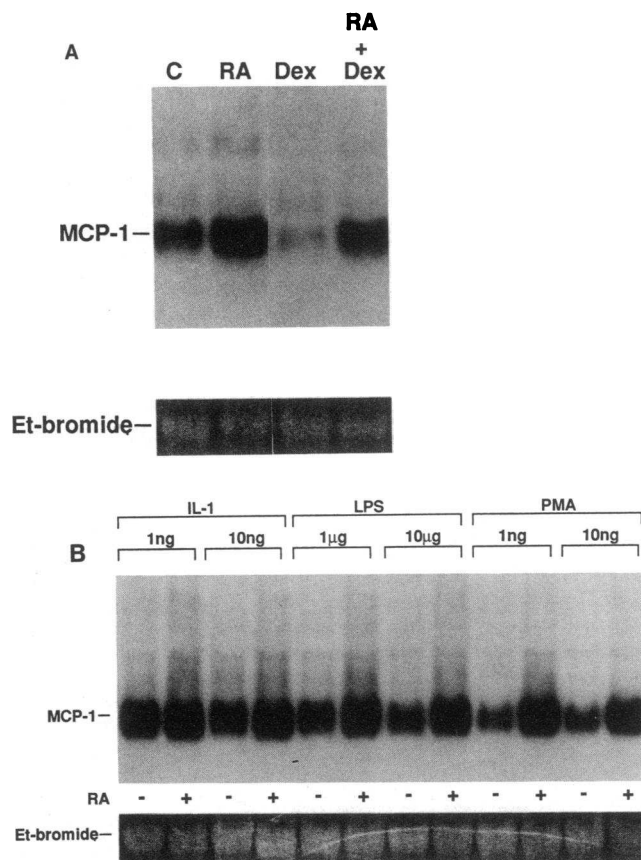


Figure 7. Dexamethasone and retinoic acid differentially affect MCP-1 expression. (A) Articular chondrocytes (P3) were cultured in DMEM + 1% FBS in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of retinoic acid (RA) for 3 d. Dexamethasone (10^{-7} M; lanes 3 and 4) was added for the last 5 h. RNA was extracted and analyzed by Northern blotting. To assess the amount of RNA per lane the filters were stained with ethidium bromide. (B) Retinoic acid increases the induction of MCP-1 mRNA by IL-1, LPS, and PMA. Chondrocytes (P3) were cultured in DMEM + 10% FBS in the presence (+) or absence (-) of retinoic acid (10^{-6} M) for 3 d. The cells were then stimulated with IL-1 (lanes 1-4), LPS (lanes 5-8), or PMA (lanes 9-12) at concentrations as indicated above the lanes for 4 h. RNA was extracted and analyzed as above.

tional significance of the heparin binding of MCP-1 has not yet been determined. It could provide a basis for storage of this cytokine in cartilage extracellular matrix. A potential role of heparin binding in modulating MCP-1 binding to its receptors as it has been shown for bFGF (30), also needs to be evaluated.

In situ hybridization experiments provided new insight into the kinetics and distribution of IL-1 effects on chondrocyte gene expression in cartilage organ cultures. Cells in the superficial tangential zone expressed MCP-1 already 2 h after stimulation with IL-1. This response was transient and mRNA levels had already decreased after 4 h. Interestingly, the response of the cells in the deeper layers of cartilage was not only delayed in onset but of much longer duration. Since the effect of IL-1 alone on MCP-1 is usually fast and brief, the prolonged expression by chondrocytes in deeper layers may be due to secondary events. One interesting hypothesis is that this may be due to the expression of TGF β which can cause a similarly long lasting increase in MCP-1 mRNA levels. In previous studies we demonstrated that IL-1 induces a late increase in TGF β 1 and a rapid increase in TGF β 3 mRNA levels in chondrocytes (5). These differences in the kinetics of MCP-1 expression may also be indicative of functional heterogeneity of chondrocytes in the different layers of cartilage.

The characterization of inducers of MCP-1 expression that are present in cartilage or synovium resulted in the identification of two peptide regulatory factors as new activators of this gene. In addition to IL-1, TNF α , LPS, PDGF, and serum, previously known inducers of MCP-1 in other cell types, we demonstrate that TGF β and LIF stimulate this cytokine in chondrocytes. Except for the induction of bone resorption in vivo and in vitro (31, 32), LIF has not yet been characterized with respect to its effects on connective tissue metabolism. The finding that LIF induces MCP-1 expression represents the first documentation that LIF can regulate the synthesis of other cytokines and that it affects chondrocyte function. We recently showed that chondrocytes and synoviocytes produce LIF and that synovial fluids contain LIF activity (24). Based on its presence in the joint, LIF is probably an important inducer of chondrocyte MCP-1 production in vivo.

TGF β is abundant in inflammatory joint fluids (33, 34). It is a potent regulator of chondrocyte function and also known to profoundly affect specific as well as nonspecific immune reactions (reviewed in 35). In cartilage TGF β has mainly anabolic functions. It promotes chondrocyte proliferation (5, 23), the synthesis of extracellular matrix (36) and it reduces IL-1 induction of collagenase (37) and the expression of IL-1 receptors on chondrocytes (38). Among its effects on immune responses, TGF β has been shown to inhibit T and B lymphocyte proliferation, to modulate immunoglobulin synthesis and to deactivate monocytes. In contrast to these antiinflammatory actions are the potent effects of TGF β as a neutrophil (39) and monocyte chemoattractant in vitro and in vivo (40, 41). In addition, we showed recently that TGF β induces production of the neutrophil chemoattractant, IL-8, by chondrocytes (4). This study has demonstrated that TGF β induces MCP-1 mRNA levels comparable to the levels seen after stimulation with IL-1. Thus, TGF β can directly and indirectly contribute to the recruitment of neutrophils and monocytes via the induction of IL-8 and MCP-1.

Two antiinflammatory steroids, dexamethasone and RA, which inhibit cytokine synthesis in other cell systems, were shown to differentially affect MCP-1 gene expression in chon-

drocytes. As has been shown for other cytokines and for MCP-1 in other cell types (25), dexamethasone inhibited MCP-1 mRNA expression. Glucocorticoids bind to intracellular receptors that recognize glucocorticoid-responsive elements in the promoter regions of genes. In addition, glucocorticoids can modulate AP-1 DNA binding activity and thereby inhibit gene expression (42). Dexamethasone abrogated MCP-1 mRNA induction after stimulation not only with IL-1 but also in response to direct activators of protein kinase A (cAMP) and the protein kinase C (PMA) signal transduction pathways.

In contrast, RA was identified as a new and potent inducer of MCP-1 gene expression in chondrocytes. The effects of RA on gene expression depend on its binding to intracellular RA receptors. The RA/receptor complexes interact with retinoic acid response elements (RARE) in the promoter region of several genes (43, 44). As for glucocorticoids, an interaction with AP-1 binding proteins has been described (45, 46). In the case of RA, the formation of unproductive complexes with c-Jun is thought to lead to a decrease in AP-1 activity (47). The promoter region of MCP-1 has recently been sequenced, but detailed analyses concerning functionally important regulatory elements are lacking. A phorbol ester responsive element (TRE) confers PMA responsiveness and this has been shown to be functionally important in fibroblasts (17). Interestingly, PMA caused only a minimal increase in MCP-1 mRNA in chondrocytes, but after preincubation of the cells for 3 d with RA, PMA induced a very strong increase in mRNA levels. In addition to the PMA response, MCP-1 induction by IL-1 and LPS was also upregulated by RA. These findings are in contrast to recent results obtained with human monocytes, where RA inhibited IL-1-induced synthesis of IL-6 (29). This suggested that either the regulation of IL-6 induction by IL-1 differs from the regulation of MCP-1 by IL-1, or that there are cell-type specific factors which account for these contrasting results. Analysis of gene expression of other cytokines (IL-6, IL-8, LIF) revealed that the stimulated expression of all these cytokines was markedly upregulated in chondrocytes by pretreatment of the cells with RA. Furthermore, AP-1 DNA-binding activity as analyzed by mobility shift assay documented that in chondrocytes RA treatment for 72 h resulted in an increase in AP-1 activity (unpublished results). Thus, the profound stimulatory effect of RA on expression of MCP-1 in chondrocytes, apparently represents a tissue specific difference in the effect of this agent on gene expression, and may be related to an increase in AP-1 activity.

In conclusion, through the production of biologically active MCP-1 in response to inducers that are present in the articular tissues of patients with inflammatory and degenerative joint diseases, cartilage can directly contribute to synovial inflammation and destruction of its extracellular matrix. Furthermore, understanding of the regulation of cytokine and growth factor-induced MCP-1 expression in cartilage should be valuable in the understanding and control of the clinical manifestations and sequelae of arthritides.

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