Interleukin (IL)-6 and Its Soluble Receptor Induce TIMP-1 Expression in Synoviocytes and Chondrocytes, and Block IL-1-induced Collagenolytic Activity*

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To define the potential role of interleukin-6 (IL-6) and its soluble receptor α in cartilage metabolism, we analyzed their effects on tissue inhibitor of metalloproteases (TIMP) synthesis by synoviocytes and chondrocytes. TIMP-1 production by isolated human articular synovial fibroblasts and chondrocytes, stimulated by IL-6 and/or its soluble receptor, was first assayed by specific enzyme-linked immunosorbent assay; the slight stimulatory effect of IL-6 on TIMP-1 production by both types of cells was markedly amplified by the addition of soluble receptor, the maximal secretion being observed only at 96 h. TIMP-1 mRNA expression, determined by ribonuclease protection assay, was induced by IL-6 together with its soluble receptor, but TIMP-2 and -3 mRNAs were not affected by these factors. A specific neutralizing antibody abolished the effects of the soluble receptor. Finally, supernatant from synoviocytes stimulated by IL-6 plus its soluble receptor blocked almost completely the collagenolytic activity of supernatant from IL-1-induced synoviocytes. These observations indicate that IL-6 and its soluble receptor have a protective role in the metabolism of cartilage. Given the high levels of soluble receptor in synovial fluid and the marked induction of IL-6 by IL-1 or TNF-α, it is likely that IL-6 and its soluble receptor are critical in controlling the catabolic effects of pro-inflammatory cytokines.

Tissue inhibitors of metalloproteases (TIMPs) are important and specific inhibitors of matrix metalloproteases (MMPs) activity (1). These two classes of molecules play a crucial role in the fine regulation of extracellular matrix turnover, which is altered in most pathological states associated with abnormal extracellular matrix formation (i.e. fibrotic diseases) or tissue destruction (i.e. rheumatoid arthritis). TIMP proteins can bind either to the active site of MMPs, thus blocking access to the substrate, or to the precursor form, blocking further activation.

So far, the sequences coding for four human TIMPs (TIMP-1, -2, and -3 and, more recently, TIMP-4) have been identified (1–11). The expression of TIMP-1 proved to be both constitutive and inducible, whereas TIMP-2 appeared to be widely expressed but not inducible (1, 12). A recent study indicates that TIMP-3 expression is also constitutive and inducible (13).

TIMP-1 expression in differentiated chondrocytes and fibroblasts has been shown to be regulated by a few growth factors or cytokines, among which transforming growth factor-β (TGF-β) is considered to be the most important inducer (1, 14). On the other hand, catabolic cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), the main promoters of MMPs synthesis and matrix degradation, have a marked inhibitory effect on TIMP-1 expression by chondrocytes, although some results have shown that IL-1, depending on the conditions, can either stimulate or inhibit the synthesis of TIMP-1 (15–19). IL-6 was initially considered a pro-inflammatory cytokine like TNF-α and IL-1, because of its IL-1-like effects on immune and hepatic cells. On the other hand, IL-6 has repeatedly been shown not to induce the synthesis of MMPs (20, 21). Instead, IL-6 can induce, in some conditions, the synthesis of TIMP-1 in human articular chondrocytes and fibroblasts (20). Because IL-6 is one of the most abundant cytokines, synthesized in large amounts in response to IL-1 or TNF-α stimulation, this effect might represent a very important protective mechanism, and be consistent with other effects of IL-6 on synthesis of inhibitor acute phase proteins such as α2-macroglobulin and IL-1 receptor antagonist (22, 23). Induction of TIMP-1 by IL-6, however, was very weak compared with that obtained with TGF-β (20, 24, 25), but the effects of IL-6 were not tested in the presence of soluble IL-6 receptor α (sIL-6Ra).

IL-6 acts on the cells through a multimeric receptor composed of an α chain (IL-6Rα or gp80) and a β chain (gp130) (reviewed in Ref. 26). Recombinant sIL-6Ra has been shown to form an active complex with IL-6 and gp130 molecules expressed on cell surface (27–30), and to act as an agonist of IL-6 functions. In particular, sIL-6Ra stimulates osteoclast formation by IL-6 (31), amplifies the synthesis of acute phase proteins (28, 32), stimulates the proliferation of human papilloma-immortalized cervical cells (33), and induces proliferation of synovial fibroblastic cells (34). In various body fluids including serum, urine, and synovial fluid, naturally occurring sIL-6Ra appears to be present in patients with various diseases as well as in healthy subjects (35–37).

The aim of the present study was therefore to analyze the effects of IL-6 and sIL6Ra on TIMP-1 production by human articular chondrocytes, in order to define their eventual role in controlling processes induced by catabolic cytokines.
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MATERIALS AND METHODS

Reagents—human IL-1, IL-6, sIL-6Rα, TGF-β1, recombinant human soluble gp130, and anti-human IL-6R neutralizing antibody were purchased from R & D Systems (Minneapolis, MN). IL-1, IL-6, sIL-6Rα, and TGF-β1 were defined by the manufacturer as >97% pure, with endotoxin levels <0.1 ng/μg. Activities (ED₅₀) defined by the manufacturer ranged between 0.5 and 1.5 ng/ml for IL-6 (proliferation of IL-6-dependent murine plasmacytoma cell line T1165.85.2.1), between 5.0 and 15.0 ng/ml for sIL-6Rα (enhancement of the IL-6-induced growth inhibition of murine M1 myeloid leukemic cells), between 0.02 and 0.06 ng/ml for TGF-β (inhibition of the murine IL-4-dependent TH1 cell proliferation), and between 0.5 and 2 ng/ml for gp130 (inhibition of the IL-6R enhancement of IL-6 activity on a mouse myeloid leukemia cell line).

Isolation of Human Synovial Fibroblasts and Articular Chondrocytes—Human synovial fibroblasts were isolated by enzymatic digestion from tissue obtained during knee joint replacement for osteoarthritis or rheumatoid arthritis as described previously (38). After digestion, synovial fibroblasts were expanded in culture flasks in Dulbecco’s modified Eagle’s medium (DMEM) containing Glutamax, pyruvate sodium, 1000 mg/liter glucose, and pyridostine (Life Technologies AG, Basel, Switzerland), supplemented with 10% heat-inactivated fetal calf serum (FCS), 10 units/ml penicillin, and 10 mg/ml streptomycin. At confluence, they were trypsinized and split before further expansion. Cells were used between passages 2 and 5.

Human articular chondrocytes were isolated by enzymatic digestion from autopsied femoral condyles as described previously (39). Only cells harvested after 3 days were used for these studies. After digestion, the cells were stored overnight at 37 °C in DMEM supplemented with 10% FCS, 10 units/ml penicillin, and 10 mg/ml streptomycin. Cells were used the next day for stimulation experiments.

TIMP-1 Protein Quantification—For stimulation experiments, 10⁵ articular chondrocytes or 2 × 10⁶ synovial fibroblasts were plated in 96-well microplates and 48-well microplates, respectively. Supernatants were harvested at the indicated times and tested for TIMP-1 protein by enzyme-linked immunosorbent assay (ELISA) using specific antibodies to TIMP-1 protein as described previously (40). The sensitivity of the protein assay was 10 ng/ml.

RNA Preparation and Analysis—A total of 10⁶ chondrocytes and synovial cells were plated in 35-mm culture dishes, and RNA was prepared 48 h after stimulation using the RNeasy total RNA kit (Qiagen AG, Basel) according to the manufacturer’s instructions. cRNA molecules overlapping human glyceraldehyde-3-phosphate dehydrogenase (319–516), TIMP-1 (92–293), TIMP-2 (157–653), and TIMP-3 (429–806) were used for RNase protection assay as described previously (41) (the positions indicated are relative to the A residue of the initiation codon). Briefly, 2 × 10⁶ cpm of each probe were hybridized to 1 μg of total RNA at 50 °C for 12 h. Unprotected RNA molecules were then digested in the presence of 3 μg/ml RNase A (Boehringer Mannheim) and 3 units/ml RNase T1 (Boehringer Mannheim). The protected molecules were separated by migration on a 8 x 4% acrylamide denaturing gel and visualized by autoradiography. The intensity of the bands was then analyzed by densitometry.

Functional Assay of TIMP Activity—Synoviocyte supernatants stimulated with IL-6 + sIL-6Rα or TGF-β were tested for TIMP activity by their ability to inhibit collagenase activity in IL-1-stimulated synovocyte supernatants; supernatants of synoviocytes stimulated by IL-6 + sIL-6Rα (50 ng/ml each) or TGF-β (10 ng/ml) were obtained after 96 h of culture as described for TIMP-1 protein quantification. Increasing concentrations of these supernatants were mixed with constant concentrations of supernatants from synoviocytes stimulated by IL-1 (20 ng/ml culture medium was used to compensate for volume differences. The mixture was assessed for collagenolytic activity by a functional collagenase assay as described (20).

Statistical Analysis—Paired two-sample Student’s t test was used to analyze the significance of the effects of IL-6 + sIL-6Rα supernatants (SNs) and TGF-β SNs on the collagenolytic activity of IL-1SNs. p < 0.05 was considered significant.

RESULTS

Effects of IL-6 and sIL-6Rα on TIMP-1 Protein Release by Human Synovial Fibroblasts—We first examined the effects of each IL-6 and sIL-6Rα alone or in combination on human synoviocytes and compared them with the effects of TGF-β. TIMP-1 protein was quantified in the supernatants of synoviocytes stimulated for 96 h. Optimal stimulatory concentrations were determined in preliminary experiments of dose dependance and found to amount to ~30 for sIL-6Rα and ~50 ng/ml for IL-6. At these concentrations, the effects of IL-6 alone on TIMP-1 production were generally present but not significant (~1.2 times the levels of control) (Fig. 1). On the other hand, the induction of TIMP-1 production by IL-6 in combination with sIL-6Rα was ~3.0-fold, even higher than that obtained with TGF-β in these conditions (~1.7-fold). sIL-6Rα alone also increased TIMP-1 production (~1.7-fold), an effect more pronounced than that of IL-6 alone. In order to confirm the role of sIL-6Rα in this synergy, we performed a set of experiments preincubating soluble receptors with a neutralizing anti-sIL-6R antibody. This antibody completely inhibited the induction of TIMP (Fig. 2). Recombinant soluble gp130, known to specifically inhibit the effects of IL-6, also inhibited the effects of IL-6 with sIL-6R; anti-sIL-6Rα antibody and gp130 had no significant effects on unstimulated cells. Differences between control values of Figs. 1 and 2 are certainly related to the fact that they are based on different cultures, performed with synoviocytes from different origins.

Kinetics of TIMP-1 Induction—To further investigate TIMP-1 expression induced by IL-6 and sIL-6Rα, we performed kinetic studies on synovial cultures (Fig. 3A). The induction was detectable after ~24 h of stimulation (~2-fold). Maximal effects on TIMP-1 production, however, were observed after 96 h of IL-6 and sIL-6Rα co-stimulation (~3.3-fold). To determine if IL-6 and sIL-6Rα have the same effects on other mesenchymal cells, similar experiments were performed on human chondrocytes. The effects proved even stronger (Fig. 3B); the induction was detectable after 48 h of stimulation (~2.2-fold) becoming more evident after 72 h (~6.2-fold). Maximal effects on TIMP-1 production were also observed after 96 h of IL-6 and sIL-6Rα co-stimulation (~29-fold). In this experiment, maximal induction by TGF-β was observed after 72 h without significant changes after 96 h of treatment (~11-fold and ~10-fold, respectively). We also observed a time-dependent increase in TIMP-1 production in unstimulated cultures, i.e. ~8.4-fold after 96 h. The time course experiment with chondrocytes was performed only once, because of the limited number of fresh chondrocyte cultures available. However the pattern is the same at each time point, and the results very similar to those obtained with synoviocytes.

Effects of IL-6 and sIL-6Rα on the Expression of TIMP mRNA—To confirm and further define the effects of sIL-6Rα with IL-6 on TIMP synthesis, mRNA expression was analyzed on synovial fibroblasts (Fig. 4). Cells were stimulated for 48 h
pressed, but at lower levels than TIMP-1. In addition, IL-6 both cell types TIMP-2 and -3 mRNA were constitutively ex-riboprobe difficult. The same analysis also revealed that in the RNA preparation used, probably as a result of the second-

ation using TIMP-1 riboprobes was variable, depending on the density of 10^5 cells/cm^2 in DMEM supplemented with 2% FCS, in the absence (lane 1) or presence of 10 ng/ml IL-6 (lane 2), or in the presence of both 10 ng/ml IL-6 and 50 ng/ml sIL-6Ra (lane 3). Total RNA (1 μg) was used for the detection of TIMP-1, -2, and -3 mRNA by RNase protection assay, and (0.5 μg) for the detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Densitometries for un-

stimulated, IL-6-stimulated, and IL-6+sIL-6R-stimulated cells were, respectively, 1.0, 0.7, and 1.8 for TIMP-1; 1.0, 0.9, and 1.0 for TIMP-2; and 1.0, 0.9, and 1.1 for TIMP-3, as analyzed by NIH image and normal-

ized for glyceraldehyde-3-phosphate dehydrogenase.

whether alone or together with sIL-6Ra did not induce TIMP-2 and -3 expression on these cells. TIMP-1 mRNA expression was also shown to be induced at 4 and 12 h (data not shown).

Functional Assessment of TIMP Activity—In order to evaluate the ability of TIMP induced by IL-6 + sIL-6Ra to inhibit collagenase, and to get an estimate of its potency, we performed a functional analysis. In these experiments, supernatants of synoviocytes stimulated with IL-6 + sIL-6Ra or TGF-β were tested for TIMP activity by their ability to inhibit collagenase activity in supernatants of synoviocytes stimulated by optimal doses of IL-1. The results (Fig. 5) showed that IL-6 + sIL-6Ra supernatants were able to suppress on an average more than 80% of the collagenolytic activity present in equal amounts of IL-1 supernatants (fourth column compared with first column, p = 0.02). This was comparable to the efficiency of TGF-β supernatants, capable on an average of inhibiting ~75% of the collagenolytic activity of supernatant from IL-1-induced cells (seventh column compared with first column, p = 0.03). Supernatants from unstimulated synoviocytes or from synoviocytes stimulated for only 24 or 48 h by IL-6 + sIL-6Ra had no significant inhibitory effect (data not shown).

**DISCUSSION**

The aim of this study was to define the effects of IL-6 and its soluble receptor on TIMP-1 expression by cells that are part of cartilage metabolism and turnover, by analyzing the response of human articular chondrocytes and synovial fibroblasts. IL-6 alone exerted a very weak effect on TIMP-1. Our principal finding is that the effect of IL-6 can be enhanced by adding sIL-6Ra to the culture medium. This increase was observed in human articular chondrocytes and human synovial fibroblasts, suggesting that the weak IL-6 effect on TIMP-1 expression was a result of the low level of IL-6Ra expressed on the surface of
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Fig. 5. Functional assessment of TIMP activity. TIMP activity in the SNs was assessed by inhibition of collagenolytic activity. SNs of synoviocytes, stimulated by IL-6 + sIL-6Ra or TGF-β, obtained after 96 h as described, were mixed at the indicated concentrations with constant concentrations of IL-1-stimulated synoviocyte SNs rich in collagenase and culture medium to compensate volume differences. The residual collagenolytic activity of the mixture was assessed by a functional collagenase assay as described. Results are expressed as percent residual activity compared with the activity of IL-1 synoviocyte SNs mixed only with medium (first column). Results represent the average of three independent experiments and error bars the standard deviations. Asterisks (⁎) indicate a p < 0.05 compared with IL-1β SNs in the absence of TGF-β or IL-6 + sIL-6Ra SNs (first column).

these cells. These results are consistent with the identification of the IL-6 response element in the rat tissue inhibitor of the TIMP-1 promoter (42).

It has already been observed that significant amounts of sIL-6Ra (20–25 ng/ml) can be present in the serum of healthy donors (35), and that serum concentrations may increase in certain pathological conditions such as interstitial pneumonia (43), multiple myeloma (44), and human immunodeficiency virus infection (45). High amounts of sIL-6Ra, equivalent to the doses used in our experiments, are also found in synovial fluid (5–40 ng/ml). Although hepatocytes seem to be a major source of sIL-6Ra in body fluids, inflammatory cells as well as human synoviocytes and articular chondrocytes have also been shown to express and release this molecule (37). It might be of relevance that synovial fluid levels appear to be higher (20–25 ng/ml) than in the joints of patients affected by inflammatory diseases than in the joints of patients with non-inflammatory diseases (38). Synoviocytes and most probably chondrocytes, at least in the superficial layers, therefore have access to sIL-6Rα by diffusion, which renders them more susceptible to the effects of IL-6.

Conflicting results have been obtained on the induction of TIMP-1 expression by IL-6 alone; some authors reported mild effects (20, 46), whereas others observed no effects at all (47). These discrepancies could be due in part to the culture conditions and/or the heterogeneity of the primary cells. Among other parameters, the origin and concentration of FCS might be an important factor. Chondrocytes are known to dedifferentiate in monolayer cultures; during the first passages of the primary culture, the cells still have a chondrocytic phenotype, but it is not clear whether they are really identical to chondrocytes in vivo. For this reason and because large amounts of chondrocytes are difficult to obtain, we performed most experiments with synovial fibroblasts, another mesenchymal cell type present in joints and involved in extracellular matrix turnover and tissue destruction.

Other cytokines of the IL-6 family, such as leukemia inhibitory factor and IL-11, were also tested. The stimulation of TIMP-1 production was comparable to that obtained with IL-6 alone but clearly weaker than that obtained by adding both IL-6 and sIL-6Ra (data not shown). A more extensive comparative study of the effects of IL-6 and related cytokines on TIMP-1 production by human articular chondrocytes was performed by Nemoto et al. (48). With the exception of oncostatin M, the effects of these factors were weak (48). Oncostatin M appeared to be a potent stimulator of TIMP-1 expression in these cells. However, taken together with the very high levels of IL-6 and sIL-6Ra in synovial fluid compared with other cytokines (38, 49), our results suggest that the combination of IL-6 and sIL-6Ra represents one of the main stimuli of TIMP-1 production by cells involved in cartilage metabolism. More specifically, because of the marked induction of IL-6 synthesis after IL-1 or TNF-α stimulation (38), IL-6 together with sIL-6Ra probably plays a specially important role in feedback mechanisms in response to the effects of these inflammatory cytokines. Interestingly, IL-6 has also been shown to enhance the synthesis of TGF-β (50), which might reinforce these feedback circuits. The contradictory results, which have shown that IL-1, depending on the conditions, can either stimulate or inhibit the synthesis of TIMP (17, 19), suggest interactions and indirect effects through other cytokine receptors systems, including IL-6/IL-6Ra/sIL-6Ra.

In addition to its effects on TIMP, IL-6 increases the hepatic synthesis of α2-macroglobulin, another inhibitor of various proteases including collagenase and stromelysin, which further demonstrates the protective role of IL-6. Overall, these data support the concept that IL-6-related cytokines, and especially IL-6 with its soluble receptor, are important in controlling the catabolic effects of proinflammatory cytokines, in particular during remodeling processes. In this context, further studies on the effects of IL-6 and sIL-6Ra on MMP expression and proteoglycan synthesis are in progress in our laboratory and might be of particular interest. In fact, it has recently been demonstrated that IL-6 + sIL-6Ra cause the induction of collagenase 3 expression in rat osteoblast cultures (51).

Finally, we have been able to demonstrate that synovial fibroblasts can express TIMP-3 mRNA. TIMP-3 was first isolated in chicken (Ch-imp-3) (6) and then in humans (2, 8, 10, 52, 53). Like TIMP-1 and -2, TIMP-3 inhibits MMP activity (6) and stimulates the division of serum-deprived cells (54). The observation that synovial fibroblasts express TIMP-1, -2 and -3 mRNAs raises the question of the relative importance of these proteins in synovium and cartilage metabolism. In synoviocytes, only TIMP-1 mRNA expression was increased by IL-6 and sIL-6Ra, suggesting that the potential protective effect of this cytokine receptor system is mainly mediated by TIMP-1. The differential regulation of TIMPs has already been described in normal and malignant cell lines (12). Moreover, structural analysis of the promoters of the corresponding three genes revealed a housekeeping-like structure for TIMP-2 and -3 gene promoters (9, 55). Both lack the classic TATA box, area rich in CG, and have multiple SP1 binding sites. On the other hand, the structure of TIMP-1 promoter possesses typical elements of inducible promoters, like phorbol ester-responsive elements, c-Fos, c-Jun, and Ets binding sites (56, 57).

In conclusion, our results suggest that the anti-catabolic response in joint tissues is prompted to a great extent by an increased expression of TIMP-1, mediated by protective cytokines among which IL-6 together with sIL-6Ra plays a critical role. The fact that TIMP-2 and -3 are unresponsive in these conditions probably means that their role is more limited to normal cartilage turnover.

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