Rheumatoid arthritis (RA) is a chronic disease characterized by sustained inflammation in synovial joints and concomitant destruction of articular cartilage and bone1. During chronic joint inflammation, considerable thickening of the lining is characteristic, and the subsynovial tissue becomes infiltrated by numerous macrophages and lymphocytes. In the early stages of RA, an unknown antigen activates T cells to release cytokines, which in turn activate macrophages and fibroblasts, triggering the immune-inflammatory cascade of synovitis2,3. The high density of activated macrophages in rheumatoid synovium4 suggests that these cells also play an important role in RA. Synovial lining macrophages are of crucial importance in the development of arthritis. When these cells are selectively eliminated from the joint by local application of “toxic” liposomes, the joint becomes markedly resistant to subsequent arthritis induction5-8. Cartilage degradation and joint destruction are often found in RA1. The underlying molecular basis for matrix degradation is believed to be dependent on the actions of a variety of proteolytic enzymes9. The matrix metalloproteinases (MMP) are a family of related enzymes with different substrate specificities, which together are capable of degrading all the components of the extracellular matrix10,11. They also contribute significantly to tissue damage in chronic inflammatory diseases such as RA. MMP are first secreted from the cells as inactive proenzymes. Activation in vivo is thought to occur by the proteolytic cleavage involving plasmin and/or MMP such as stromelysin10. Once activated, the control of enzyme activity is dependent on the local concentration of tissue inhibitors of metalloproteinases (TIMP) and on nonspecific proteinase inhibitors such as α2-macroglobulin.

Although the macrophage seems to play a cardinal role in established RA12, T lymphocytes are likely to be essential for the initiation of rheumatoid synovitis. T cell secreting interleukin 17 (IL-17) is among the first to be activated during the immune response13, suggesting that this cytokine

Modulation of TIMP-1 Synthesis by Antiinflammatory Cytokines and Prostaglandin E2 in Interleukin 17 Stimulated Human Monocytes/Macrophages

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ABSTRACT. Objective. To examine the regulation of tissue inhibitor of metalloproteinase 1 (TIMP-1) synthesis by interleukin 17 (IL-17) stimulated human monocytes/macrophages in primary culture in the presence of prostaglandin E₂ (PGE₂) and antiinflammatory cytokines, and to compare this with the regulation of matrix metalloproteinase (MMP-9) production.

Methods. IL-17 stimulated human monocytes isolated from the peripheral blood of healthy donors were cultured in the presence of PGE₂, cyclic adenosine monophosphate (cAMP) mimetics (IBMX, cAMP, forskolin, cholera toxin), or antiinflammatory cytokines (IL-4, IL-10, IL-13), or with protein kinase inhibitors of diverse specificity. MMP-9 and TIMP-1 were measured using specific ELISA, while expression of specific messenger RNA was determined by Northern blotting.

Results. IL-17 stimulated an increased level of MMP-9 production relative to TIMP-1 production in monocytes/macrophages. Stimulation was accompanied by upregulation of specific MMP-9 mRNA expression relative to TIMP-1 mRNA. Exogenous PGE₂, cAMP, and cAMP-mimetics completely inhibited both basal and IL-17 induced MMP-9 synthesis, while only IL-17 induced TIMP-1 synthesis was abrogated. The same effect was found for the antiinflammatory cytokines. Both basal and IL-17 induced production of TIMP-1 involved p42/44 and p38 kinases and nuclear factor κB signaling pathways.

Conclusion. The excess of MMP-9 over TIMP-1 production, and decreased inhibition of MMP-9 activity in chronic rheumatoid diseases, may result in cartilage degradation and joint destruction. (J Rheumatol 2001;28:712–8)

Key Indexing Terms: INTERLEUKIN 17 REGULATION TISSUE INHIBITOR OF METALLOPROTEINASE 1 MATRIX METALLOPROTEINASE 9 MONOCYTES/MACROPHAGES
may play an important role in the early stages of inflammation. We and others have reported that biologically active IL-17 was highly produced by RA but not osteoarthritis (OA) synovium, and the concentration of this cytokine was significantly elevated in the synovial fluid (SF) of patients with RA compared with OA.14,15 We described the potential of macrophages to synthesize MMP-9 upon stimulation by IL-17.14 MMP-9 was found at high levels in the SF of patients with RA.16-19 Abnormally high levels of this MMP may contribute to destructive processes of the joints in patients with RA; a correlation was found between the increased level of 92 kDa gelatinolytic activity in RA SF and disease severity.17-19 The activity of MMP-9 is thought to be regulated by activation levels of the proenzyme and inhibition by TIMP-1. Not only is TIMP-1 a potent inhibitor for gelatinase B, but the gelatinase B/TIMP-1 complex is also highly resistant to activation by stromelysin.20 Latent MMP-9 and/or MMP-9/TIMP-1 complexes were shown to be elevated in RA SF compared with OA SF.19

We observed that IL-17 stimulated macrophages to increase TIMP-1 secretion and that this production was differentially regulated by antiinflammatory cytokines and prostaglandin E2 (PGE2) compared with the regulation of MMP-9.

**MATERIALS AND METHODS**

*Monocyte/macrophage isolation from human blood.* Peripheral blood mononuclear cells (PBMC) consisting of lymphocytes and monocytes were isolated from heparinized blood samples from healthy donors (n = 24; mean age 35 yrs, range 26–52) as described.11 Briefly, the samples were diluted 1:2 with phosphate buffered saline (PBS) containing 3 U/ml preservative-free heparin (Sigma Diagnostic, St. Louis, MO, USA), centrifuged over Ficoll-Hypaque (Amersham Pharmacia Biotech, Baie d’Urfé, Québec, Canada), and washed 3 times in RPMI 1640 (Gibco-BRL, Life Technologies Inc., Gaithersburg, MD, USA) containing penicillin and streptomycin (100 U/ml, 100 µg/ml, respectively), and supplemented with 2 mM L-glutamine (Gibco-BRL). All reagents were endotoxin tested. The cells were seeded in untreated plastic petri dishes (Flow Laboratories Inc., McLean, VA, USA) at a density of 4 × 10^6 cells/cm^2. They were allowed to adhere to plastic dishes (60 min at 37°C) in a humidified atmosphere and 5% CO₂ in RPMI medium.21 The nonadherent cells (mainly lymphocytes) were removed by vigorous washes with PBS. Monocytes/macrophages represented > 92% of the cell population, determined by flux cytometry analysis (Becton Dickinson, Mountain View, CA, USA) using an anti-CD14 antibody (dilution 1:250; ID Labs Inc., London, ON, Canada). Cell viability was determined by trypan blue exclusion and was > 95%.

*Culture conditions.* For dose-response studies, monocytes/macrophages were cultured in 12 well plates (Corning Inc., Corning, NY, USA) in RPMI supplemented with 2.5% heat inactivated fetal calf serum (FCS) at 37°C in 5% O₂/5% CO₂ for 72 h. The nonadherent cells (mainly lymphocytes) were removed by vigorous washes with PBS. Monocytes/macrophages were cultured for increasing periods of time (0, 12, 24, 48, 72 h) in the presence of 100 ng/ml rhIL-17. Supernatants were collected and the levels of MMP-9 and TIMP-1 determined.

To examine the effects of antiinflammatory cytokines, the monocytes/macrophages were stimulated for 72 h with 100 ng/ml rhIL-17 in the absence or presence of 10 ng/ml rhIL-4, rhIL-10, and rhIL-13 (R&D Systems). The supernatants were collected, and the levels of TIMP-1 determined.

The effect of the exogenous PGE2, and different cAMP-mimetics on MMP-9 and TIMP-1 production was examined by adding PGE2 (2 µg/ml; Sigma), isoosyn-1-methylxanthine (IBMX; 200 µM), forskolin (60 µM), cholera toxin (100 ng/ml), and 8-bromo-adenosine-3′,5′-cyclic monophosphate (10 to 200 µM), all from Calbiochem, to the culture of 72 h IL-17 stimulated cells. Cell culture supernatants were recovered and MMP-9 and TIMP-1 levels determined.

To explore the intracellular signaling pathways activated by IL-17 in monocytes/macrophages and involved in TIMP-1 synthesis, we looked at the effects of cell-permeable protein kinase inhibitors of diverse specificity. Monocytes/macrophages were pretreated 30 min with the following agents prior to the addition of 100 ng/ml rhIL-17 for 72 h at 37°C: PD98059 (50 µM; Upstate Biotechnology, Lake Placid, NY, USA), SB202190 (10 µM; Calbiochem), and nuclear factor-κB (NF-κB) inhibitor, PDTC (30 µM; Calbiochem). Cell culture supernatants were recovered and TIMP-1 levels determined.

For all experiments, the proliferation of cells was measured by 3H-thymidine incorporation, cells were counted, and cell viability was determined by trypan blue exclusion to exclude the possible toxic effect of the inhibitor used.

**TIMP-1 and MMP-9 detection.** Measurements of TIMP-1 and MMP-9 were performed using ELISA kits (Amersham) according to the manufacturer’s instructions. The ELISA kit for MMP-9 specifically detected the proMMP-9, while that for TIMP-1 recognized total human TIMP-1, i.e., free TIMP-1 and that complexed with MMP. The detection limits were 0.6 and 1.25 ng/ml, respectively.

**Northern blotting.** Total cellular RNA was extracted with Trizol™ reagent (Gibco-BRL) as described. Following solubilization of the RNA pellet in DEPC treated sterile H₂O, RNA was quantitated spectrophotometrically at 260 nm, and the OD₂₆₀/OD₂₈₀ was between 1.7 and 2.0 with no detectable genomic DNA contamination as judged by agarose gel electrophoresis. For Northern blot experiments, generally 5 µg total RNA was resolved on 1.2% formaldehyde-agarose gels and transferred electrophoretically to nylon membranes (Hybond-N; Amersham) in 10 mM sodium acetate buffer, pH 7.8, 20 µM Tris, and 0.5 mM EDTA overnight at 4°C. The RNA was crosslinked to the membranes by exposure to ultraviolet light, and hybridized to 10 µg/ml of the 92 kDa gelatinase or TIMP-1 DNA probe overnight at 50°C.

The plasmid encoding the 92 kDa gelatinase gene (kindly provided by Dr. G. Goldberg, Washington University, St. Louis, MO, USA) was digested with BamHI and XbaI to obtain a 0.56 kb probe, as described. The human TIMP-1 cDNA probe (0.8 kb inserted into EcoRI sites of Bluescript) was kindly provided by Dr. R.H. Pang (Creative Biomolecules, Hopkinton, MA, USA). The DNA probes were labeled with digoxigenin (Boehringer Mannheim, Laval, Québec, Canada) according to the manufacturer’s specifications. Stringent serial posthybridization washes were conducted at 68°C, with the final wash in 0.1 × SSC, 0.1% SDS. Detection was carried out by chemiluminescence with CDP-Star substrate (Boehringer Mannheim) and exposure to Kodak X-AR5 film.

**Data analysis.** Values were expressed as mean ± SEM, where “n” refers to the number of different individuals. Statistical significance was assessed using Student’s t test. Significant differences were confirmed only when p < 0.05.

**RESULTS**

*Effects of IL-17 on macrophage TIMP-1 and MMP-9 synthesis.* To compare the effect of IL-17 on TIMP-1 and MMP-9 synthesis, dose and time response experiments were performed. There was a spontaneous (basal) release of MMP-9 and TIMP-1 by nonstimulated monocytes/macrophages that increased over time (Table 1). Of note, the level of spontaneously secreted proMMP-9 in 72 h culture supernatants (19.13 ± 2.28 ng/ml) was far less than the level
of spontaneously secreted total TIMP-1 (137.42 ± 6.22 ng/ml). For proMMP-9, the spontaneous secretion was always < 50% of IL-17 stimulated proMMP-9. The possible effect of low levels of endotoxin on this spontaneous MMP-9 and TIMP-1 production was overcome by using polymixin B (10 µg/ml; data not shown). In 12 h cultures there was a 4.7-fold increase in TIMP-1 production by IL-17 stimulated cells compared with the basal value. MMP-9 was not detected. In 24, 48, and 72 h cultures, increased MMP-9 production occurred (4.1, 2.6, and 3.0-fold, respectively) relative to TIMP-1 production (2.0, 1.8, and 1.4-fold, respectively).

The TIMP-1 synthesis induced by IL-17 was dose dependent (Figure 1). Previously we had found that IL-17 at the highest concentration (200 ng/ml) induced about a 3-fold increase in the level of MMP-9 compared with the basal value. In the present work, 200 ng/ml IL-17 increased basal TIMP-1 production by 1.7-fold. TIMP-1 increased synthesis was related to upregulation at the gene expression level, as indicated by the increased levels of TIMP-1 messenger RNA (Figure 2). The maximal levels of TIMP-1 mRNA were found after 3 h stimulation with IL-17. At that time, TIMP-1 mRNA and MMP-9 mRNA (data not shown) were 1.4-fold and 1.5-fold higher than in the nonstimulated cells, respectively.

**Effect of PGE2 and cAMP-mimetics on MMP-9 and TIMP-1 synthesis.** We previously reported that IL-17 stimulated the expression of cyclooxygenase 2 (COX-2) in a time dependent manner and strongly induced PGE2 secretion. We have also shown that IL-17 induced MMP-9 production in human monocytes/macrophages was dependent on endogenous PGE2 synthesis. In these experiments, we examined the effect of exogenous PGE2 and cAMP-mimetics on MMP-9 and TIMP-1 production. We found that exogenous PGE2 (2 µg/ml) almost completely inhibited both IL-17

### Table 1. Time course of MMP-9 and TIMP-1 secretion by IL-17 stimulated macrophages (100 ng/ml). MMP-9 and TIMP-1 in culture supernatants were measured by ELISA. Mean ± SEM (n = 6).

<table>
<thead>
<tr>
<th>Incubation Time, h</th>
<th>MMP-9, ng/ml Nonstimulated Cells</th>
<th>IL-17 Stimulated Cells</th>
<th>TIMP-1, ng/ml Nonstimulated Cells</th>
<th>IL-17 Stimulated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>0</td>
<td>0.07 ± 0.07</td>
<td>7.94 ± 2.04</td>
<td>37.43 ± 3.35</td>
</tr>
<tr>
<td>24</td>
<td>0.88 ± 0.13</td>
<td>3.61 ± 0.52*</td>
<td>31.05 ± 0.71*</td>
<td>61.67 ± 8.70</td>
</tr>
<tr>
<td>48</td>
<td>10.50 ± 1.42</td>
<td>27.58 ± 1.05*</td>
<td>80.27 ± 5.96*</td>
<td>146.34 ± 3.88*</td>
</tr>
<tr>
<td>72</td>
<td>19.13 ± 2.28</td>
<td>58.29 ± 5.29*</td>
<td>137.42 ± 6.22*</td>
<td>194.36 ± 7.40*</td>
</tr>
</tbody>
</table>

*p < 0.05 versus control (12 hour cultured cells), Student’s t test.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Dose-dependent stimulation of TIMP-1 by IL-17. Cells were incubated in the absence (−) or presence (+) of rhIL-17 (0–200 ng/ml) for 72 h at 37°C. TIMP-1 was determined in the culture medium by ELISA. Mean ± SEM (n = 3). *p < 0.05 versus control (unstimulated cells); Student t test.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Time course of IL-17 induction of TIMP-1 gene expression. Cells were incubated in the absence (−) or presence (+) of 100 ng/ml IL-17 for increasing periods of time (0–5 h). RNA was extracted and subjected to Northern blotting. Mean ± SEM (n = 5). Statistical analysis by Student t test, comparing values with the autologous control. Top panel: representative time course of IL-17 induction of TIMP-1 gene expression.
induced and basal MMP-9 production, while in the case of TIMP-1, only induced levels were inhibited (Figure 3). Of note, the concentration of exogenous PGE₂ used in these experiments is unphysiologically high. Additional experiments are being conducted in which dose dependent studies with physiological concentrations of PGE₂ are tested to elucidate the clinical relevance of this effect. Similar results were obtained using the cAMP-mimetics IBMX plus forskolin and cholera toxin. These results showed that the increased endogenous PGE₂ or cAMP concentration in IL-17 stimulated human monocytes/macrophages resulted in complete inhibition of MMP-9 protein synthesis, and inhibited stimulated TIMP-1 secretion. Spontaneous TIMP-1 production was not altered. These findings were confirmed by experiments in which the cAMP was added to cultures of IL-17 stimulated monocytes/macrophages (Figure 4). A dose-response inhibition of both MMP-9 and TIMP-1 was observed. Once again, spontaneous TIMP-1 production could not be inhibited by the increased intracellular cAMP concentration, indicating different mechanisms of MMP-9 and TIMP-1 regulation by PGE₂ and cAMP.

Effects of antiinflammatory cytokines on TIMP-1 synthesis. Ambient cytokines may influence the IL-17 effect on macrophages in vivo. We investigated whether antiinflammatory cytokines could modulate the IL-17 stimulated synthesis of TIMP-1. As illustrated in Figure 5, all 3 antiinflammatory cytokines inhibited IL-17 induced TIMP-1 secretion and, slightly but not significantly, the basal TIMP-1 production.

Effects of protein kinase inhibitors on TIMP-1 synthesis. We used different inhibitors of intracellular protein kinases to delineate postreceptor signaling pathways activated by IL-17 and involved in TIMP-1 synthesis (Figure 6). We found that specific inhibition of mitogen activated protein kinases (MAPK), the MEK-1/MEK-2 and p38, abrogated basal and IL-17 induced TIMP-1 synthesis, implicating these cascades in both basal and IL-17 stimulated signal transduction in macrophages.
DISCUSSION

Erosive synovitis, cartilage degradation, and joint destruction are often seen in chronic rheumatoid diseases such as RA. A variety of proteolytic enzymes elicited by both soft and hard tissue elements, as well as by inflammatory cells, are thought to be responsible for matrix degradation. Monocytes are recruited to the site of tissue injury or chronic inflammation by cell derived cytokines and chemotactic factors. Once at the site, activation of monocytes mediates the destruction of connective tissue. The activated monocytes/macrophages orchestrate connective tissue destruction either directly by the secretion of elevated MMP or indirectly by the production of proinflammatory cytokines including tumor necrosis factor-α (TNF-α) and IL-1. We and others have reported that IL-17 concentration was significantly elevated in RA SF. We have shown that IL-17 strongly stimulated the secretion of the proinflammatory cytokines TNF-α and IL-1β in human macrophages and increased the production of MMP-9. Secreted cytokines can activate other cell types, for example fibroblasts, amplifying the pathogenetic cascade. On the other hand, the increased level of MMP-9 in RA SF may contribute to destructive joint processes.

We observed that monocytes/macrophages spontaneously produced measurable levels of TIMP-1. The spontaneous secretion of TIMP-1 by unstimulated macrophages is likely to have a physiological role in maintaining tissue homeostasis. This basal production was increased when cells were stimulated with IL-17. At 72 h, IL-17 induced 3-fold and 1.7-fold increases in the level of MMP-9 and TIMP-1, respectively, compared with basal values. Many cytokines are present in arthritic joints, and it seems reasonable that they could stimulate the production of both MMP and TIMP. Spontaneous production of MMP-9 and TIMP-1 is likely to be IL-1β and TNF-α independent, while these cytokines are not present in the cultures of unstimulated cells. The stimulatory effect of IL-17 on MMP-9 synthesis has been shown to be TNF-α dependent, whereas blocking of IL-1β had no effect. Additional experiments with neutralizing antibodies against IL-1β and TNF-α will determine whether the IL-17 effect on TIMP-1 was also secondary to the release of these inflammatory cytokines. MMP-9 is secreted by macrophages as a complex with TIMP. TIMP-1 is the best known of the 4 closely related TIMP. It is a 28 kDa glycosylated protein containing 6 disulfide bonds that binds tightly but noncovalently to active MMP to form an inactive enzyme inhibitor complex. Our results show that in IL-17 stimulated monocytes/macrophages, an increased level of MMP-9 production relative to TIMP-1 production occurred. An increase of MMP activity relative to TIMP activity has been identified at sites of cartilage destruction, compared with normal surrounding cartilage. The RA plasma and RA SF concentration of MMP-9 has also been shown to be higher than levels of free TIMP-1. These data are consistent with the premise that there is an imbalance in the expression of MMP and endogenous TIMP in arthritis, and that the balance between MMP and TIMP is important in maintaining joint cartilage homeostasis.

It was observed that, in addition to MMP, TIMP production under basal conditions or after agonist stimulation was regulated by PGE synthesis in human tissue macrophages. We found that the exogenous PGE2 and cAMP-mimetics, as well as cAMP, completely inhibited both basal and IL-17 stimulated MMP-9 production, while only the former TIMP-1 secretion was abrogated. Although basal MMP-9 and TIMP-1 production depends on the COX activity, their regulation in IL-17 stimulated cells may be contingent upon separate events, as suggested by Millis, et al. The effect of exogenous prostaglandins on immune system cells is very complex. The macrophage-lymphocyte immune cell system, under physiological conditions, is “adapted” to the basal PGE2 concentration. Generally, an adaptation mechanism is responsible for the effect of exogenous concentrations. This means that an increase or decrease of endogenous levels of PGE2 in chronic disease may possibly be capable of changing the immune responses.

In human rheumatoid synovial fibroblasts, PGE down-regulates the production of TIMP-1 and proMMP-1. It was
reported that PGE suppressed the production of proMMP-9 in rabbit articular chondrocytes\(^4\), and PGE-induced cAMP downregulated IL-1-induced synthesis of MMP in human uterine cervical fibroblasts\(^4\). Pharmacological concentrations of COX inhibitors and exogenous PGE\(_2\) suppressed TIMP-1 and proMMP-1\(^45\). On the other hand, it has been shown that indomethacin suppressed the agonist induced production of TIMP and collagenase in human macrophages\(^2\), but induced collagenase in synovial fibroblasts to a high degree\(^48,49\). Thus, the effects of COX inhibitors and PGE on the production of TIMP and MMP appear to vary with the cell type.

It is now well known that cytokines function in conjunction with positive and negative feedback loops acting through pro and antiinflammatory mediators\(^50\). We have reported that IL-4 and IL-13 inhibited both basal and IL-17 induced MMP-9 production by human monocytes, while IL-10 inhibited only the former\(^14\). We report the absence of control of basal TIMP-1 production by antiinflammatory cytokines; those antiinflammatory cytokines downregulated only the IL-17 induced TIMP-1 synthesis. Even in the presence of IL-4, IL-10, or IL-13, substantial basal TIMP-1 synthesis occurred. These results once again suggest that basal TIMP synthesis may proceed via a pathway that is regulated differently by prostaglandin and antiinflammatory cytokines, compared to the basal MMP-9 synthesis. However, because specific inhibitors of mitogen activated protein kinases, namely MEK-1/MEK-2 and p38 as well as the NF-xB inhibitor, abrogated basal and IL-17 induced TIMP-1 synthesis, it is believed that these cascades are involved in both IL-17 stimulated signal transduction and basal activation.

MMP play a significant role in tissue morphogenesis and in chronic inflammatory lesions. In normal tissue, the levels of TIMP and other proteinase inhibitors are sufficient to inhibit any MMP activity. We have shown that TIMP can inhibit the autoactivation of MMP\(^41\), and it is now accepted that the balance between MMP and TIMP is important in maintaining joint cartilage homeostasis. It has been shown that the proMMP concentration in RA SF is much higher than that of TIMP\(^50\). We found that, compared to the upregulation of MMP-9, TIMP-1 was modestly stimulated by IL-17 in human monocytes/macrofages. However, both basal and IL-17 stimulated MMP-9 production was found to be sensitive to downregulation by prostaglandin and antiinflammatory cytokines, while only induced TIMP-1 synthesis responded to this type of control. Therefore, in a chronic rheumatoid disease such as RA, excessive MMP activity over TIMP activity in the invading pannus, periarticular tissue, or SF occurs as a result of increased MMP production relative to TIMP secretion. This may be the consequence of alteration in the balance between the proinflammatory and antiinflammatory cytokines and cytokine inhibitors in affected tissue.

REFERENCES


