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The effects of cytokines on metalloproteinase inhibitors (TIMP) and collagenase production by human chondrocytes and TIMP production by synovial cells and endothelial cells

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SUMMARY

It has been suggested that IL-1 produces cartilage matrix degradation by metalloproteinases such as collagenase, and that such degradation is regulated by metalloproteinase inhibitors (TIMP). Therefore, the balance between collagenase and TIMP is an important factor for tissue destruction in inflammatory joints. In the present study the effects of cytokines on collagenase and TIMP production in chondrocytes as well as the effects of cytokines on TIMP production in connective tissue cells were studied. IL-1β inhibited TIMP production in endothelial cells while enhancing TIMP production in synovial cells and chondrocytes. In addition, tumour necrosis factor-alpha (TNF-α) significantly inhibited and IL-6 significantly enhanced TIMP production in endothelial cells, synovial cells and chondrocytes. In the chondrocyte supernatant, collagenase activity/TIMP ratio was significantly elevated by the addition of either IL-1β or TNF-α to the cells, whereas the ratio was significantly decreased by IL-6. These results suggest that the cytokine effects on TIMP production are different among the different cell types, and that either IL-1β or TNF-α induce cartilage matrix degradation by disrupting the collagenase/TIMP balance, while, on the other hand, IL-6 protects the tissue through an opposite effect.

Keywords collagenase metalloproteinase inhibitors chondrocytes synovial cells IL-6

INTRODUCTION

In diseases affecting connective tissue, such as rheumatoid arthritis (RA) and collagen diseases, there is a decreased synthesis and an accelerated breakdown of the constituents of the affected tissues, including the collagens, proteoglycan, gelatin, and fibronectin, so that the connective tissue sustains damage. The enzymes associated with the breakdown of the connective tissue matrix contain metals that are called matrix metalloproteinases (MMP). Tissue cells such as synovial cells, chondrocytes and endothelial cells are known to play an important role in the destruction of joints in RA by producing IL-1 and prostaglandin-E_2. These cells also produce MMP and superoxide anions that destroy the connective tissue matrix in response to cytokines such as IL-1β and tumour necrosis factor-alpha (TNF-α) [1,2]. One MMP is a latent collagenase called MMP-1, which decomposes collagens, an important constituent of connective tissue, but has little hydrolytic effect on other constituents such as gelatin and proteoglycan. A second MMP (MMP-2) is called gelatinase because it decomposes gelatin, and also exists in an active form (mol. wt 65 000) and a latent form (mol. wt 72 000). This enzyme has no action on proteoglycan, but it is suggested that the enzyme also decomposes native type IV and type V collagen and elastin. The important third MMP (MMP-3) decomposes proteoglycan, laminin and fibronectin. MMP-3 was first isolated from synovial fibroblasts obtained from RA patients. Later it was found to be the same substance as proteoglycanase isolated from rabbit bone and stromelysin isolated from rabbit synovial fibroblasts. It is known that cells which produce MMP also produce MMP inhibitors. Collagenase inhibitor (mol. wt 30 000) has been previously extracted from fetal rabbit bone through gel filtration, and it has become apparent that this inhibitor is synthesized by various tissues in the rabbit [3]. The inhibitor was found to inhibit not only collagenase but also MMP of bone origin which decompose proteoglycan and gelatin. This glycoprotein inhibitor has thus been designated a 'tissue inhibitor of MMP' (TIMP) [4]. All the MMP mentioned above form high-affinity complexes at a 1:1 ratio with TIMP, so that their MMP activities are inhibited.

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TIMP-like inhibitors have been detected in the various tissues, body fluids and cell culture supernatants of cows, pigs, rabbits and humans. Their molecular masses all seem to differ to some extent, but are generally in the neighbourhood of 30 kD [5]. The most important factor is the MMP/TIMP ratio, since tissue destruction increases when it is high and decreases when it is low. It appears that the ratio increases and decreases according to the type of cytokine involved. Therefore, it seems advisable to make simultaneous measurements of both MMP activity and TIMP concentration.

We previously conducted investigations using *in vitro* models of cultured endothelial cells, synovial cells and chondrocytes in order to elucidate the mechanism of tissue destruction [1,2], but little work has been done to determine the effects of cytokines on TIMP production by these cells. In this study, the effects of cytokines on TIMP production by endothelial cells, synovial cells and chondrocytes were determined, and the effects of cytokines on collagenase/TIMP production were investigated.

MATERIALS AND METHODS

Culture of endothelial cells

Endothelial cells were isolated from the veins of a human umbilical cord using collagenase (from *Clostridium histolyticum*, type 1; Sigma), suspended in RPMI 1640 supplemented with antibiotics, L-glutamine and 20% fetal bovine serum (FBS), and cultured in 10-cm Petri dishes (Falcon) [6]. After the cells reached confluency, the cells were harvested with 0·25% trypsin (from Porcine pancreas; Chiba) and resuspended at 1×10^5/ml in a culture medium. Then 0·4-ml aliquots were plated on to 24-well plates (Falcon). After 24 h of incubation, the supernatant was discarded and 0·4 ml of fresh medium was added with various concentrations of IL-1β (Otsuka Pharmaceutical Co., 2×10^7 U/mg, 1×10^6 U/ml), TNF-α (Dainippon Pharmaceutical Co., 3×10^6 U/mg, 1×10^6 U/ml), or IL-6 (obtained through the courtesy of Dr Toshio Hirano, Osaka University; 5×10^6 U/mg) [7]. All of the cytokines used were from human recombinant products. The supernatant was harvested after 48 h of incubation and stored at -80°C.

Culture of synovial cells and chondrocytes

Synovial cells were cultured as previously described [1,2,8]. The synovial tissue obtained from RA patients at the time of knee replacement was minced and enzymatically dissociated using 0·2% collagenase (from *Cl. histolyticum*, type I; Sigma) and 0·25% trypsin (from porcine pancreas). The enzyme-dissociated cells were washed, suspended in RPMI 1640 with 10% FBS and cultured in 10-cm Petri dishes. The synovial cells proliferated rapidly and the dishes were stripped with trypsin for subculture. The synovial cells taken at the second and third subcultures were used in this study. The cells obtained consisted mostly of synovial fibroblasts. Similarly, slices of cartilage obtained from RA patients at surgery were enzymatically digested to isolate the chondrocytes [1,2,9]. The preparation was washed, resuspended in medium and cultured in 10-cm Petri dishes. All experiments were performed with confluent cells which had been subcultured in 10-cm Petri dishes. The experiments with chondrocytes were performed with cells at the first subculture. These cells were suspended at 1×10^5/ml in the culture medium and 0·4-ml aliquots were plated in 24-well dishes. The culture supernatants were discarded after 3–5 days and replaced with 0·4 ml of fresh medium plus various concentrations of cytokines as mentioned above. The supernatants were harvested after 72 h of incubation and stored at -80°C for the TIMP assay. For the collagenase assay, the chondrocytes were cultured for 72 h in Hanks' balanced salt solution (HBSS) in the presence or absence of cytokines, and the supernatants were stored at -80°C.

Determination of TIMP

The determination of the TIMP levels was done by the one-step sandwich EIA method using MoAbs [10,11]. A sample containing TIMP was diluted with buffer to a final volume of 0·3 ml, and was incubated with one antibody-coated polystyrene ball at 30°C for 60 min with continuous shaking (first reaction). After incubation, the ball was washed three times with a 10 mM phosphate buffer pH 7·0, containing 0·1 M NaCl, and was then incubated with continuous shaking at 30°C for 60 min with 0·3 ml (20 ng) of MoAb (Fab')-peroxidase in 10 mM phosphate buffer containing 1% bovine serum albumin (BSA) and 0·1 M NaCl (second reaction). The ball was washed again three times with buffer, and then any peroxidase activity that bound to the ball was assayed at 20°C for 3 min in 0·3 ml of 0·1 M acetate buffer, pH 5·5 containing 0·7 mM 3,3',5,5'-tetramethyl benzidine and 0·01% H_2O_2. The reaction was stopped by the addition of 0·6 ml of 1·33 N H_2SO_4 (final 1 N), and absorbance at 450 nm was measured.

Assay of collagenase

Collagenase activity was measured by collagenolytic activity using FITC-labelled collagen as a substrate (Collagenofit CLN-100, COSMOBIO). The samples (0·2 ml) were incubated at 35°C for 2–6 h with 0·2 ml of the substrate (0·1%/0·01 M acetate) in glass tubes, then 0·01 ml of 80 mM *o*-phenanthroline/50% ethanol was added to the reaction mixture to stop the reaction. The mixture was incubated at 35°C for 30 min to generate the degraded collagen. Then, 0·4 ml of 70% ethanol/0·17 M Tris HCl buffer, 0·67 M NaCl was added to the glass tubes, and incubated by agitation at 4°C. The glass tubes were centrifuged at 1750 ***g*** for 10 min, and the supernatants were measured fluorometrically at 520 nm (Em)/495 nm (Ex). Purified collagenase (type I, Sigma) was also measured to obtain total collagenase activity. Collagenase activity was calculated by the following formula: (sample − blank) ÷ (total − blank) × 120 (μg) × ((1 ÷ incubation time (min)) × (1 ÷ sample volume)).

Statistical analysis

The data were expressed as the amount of collagenase activity or TIMP produced by $6–8 \times 10^4$ endothelial cells, $2–2{\cdot}5 \times 10^5$ synovial cells and $1{\cdot}5–2 \times 10^5$ chondrocytes. Statistical significance was calculated by Student's *t*-test.

RESULTS

TIMP production by the endothelial cells and the effects of cytokines

When the TIMP production of the endothelial cells was investigated by analysing the supernatant obtained after 48 h of incubation, the addition of IL-1β (5·6, 33·3 and 200 U/ml) produced a significant decrease in the TIMP levels compared with the control ($2{\cdot}0 \pm 0{\cdot}13$ ng/ml, means ± s.d., $n = 6$). The endothelial cell TIMP production when 140, 833 and 5000 U/ml

Table 1. Effects of cytokines on TIMP production by endothelial cells

	TIMP produced by endothelial cells (ng/ml)
No cytokine	2·0 ± 0·13
IL-1β (U/ml)	
5·6	1·63 ± 0·12***
33·3	1·6 ± 0·35*
200	1·63 ± 0·14***
TNF-α (U/ml)	
140	1·58 ± 0·22***
833	1·33 ± 0·2***
5000	1·3 ± 0·13***
IL-6 (U/ml)	
2·8	2·71 ± 0·43**
16·7	2·9 ± 0·36***
100	3·0 ± 0·35***

Endothelial cells were cultured in 24-well dishes with or without cytokines for 48 h and the supernatants were measured for TIMP by sandwich EIA. The data expressed as the amounts of TIMP produced by $6–8 \times 10^4$ cells in each well are the mean ± s.d. of four replicate experiments. Statistical significance calculated by Student's *t*-test: $*P<0·05$; $**P<0·02$; $***P<0·01$ *versus* no cytokine.

Table 2. Effects of cytokines on TIMP production by synovial cells

	TIMP produced by synovial cells (ng/ml)
No cytokine	20·0 ± 2·3
IL-1β (U/ml)	
5·6	28·1 ± 3·96***
33·3	28·3 ± 3·19***
200	23·7 ± 1·98*
TNF-α (U/ml)	
140	18·7 ± 1·4
833	19·0 ± 4·1
5000	13·0 ± 1·8****
IL-6 (U/ml)	
2·8	27·7 ± 5·4**
16·7	32·7 ± 4·8****
100	34·7 ± 4·1****

Synovial cells were cultured in 24-well dishes with or without cytokines for 72 h, and the supernatants were measured for TIMP by sandwich EIA. The data expressed as the amounts of TIMP produced by $2–2·5 \times 10^5$ cells in each well are the mean ± s.d. of six replicate experiments. Statistical significance calculated by Student's *t*-test: $*P<0·05$; $**P<0·02$; $***P<0·01$; $****P<0·001$ *versus* no cytokine.

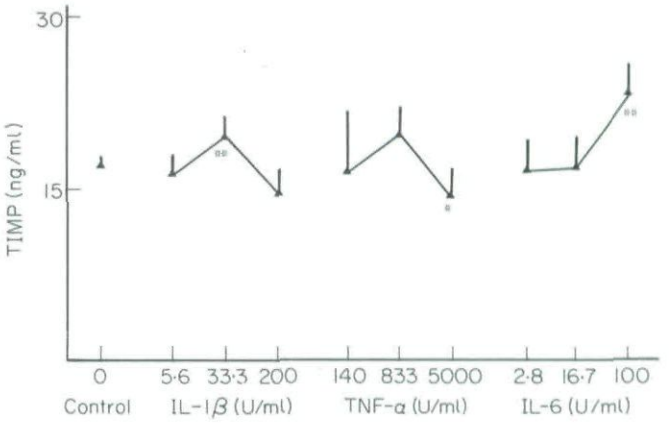

Fig. 1. Production of TIMP by chondrocytes and the effects of cytokines. Chondrocytes were cultured in a medium supplemented with 10% fetal bovine serum (FBS) either with or without IL-1β, as well as with or without tumour necrosis factor-alpha (TNF-α) or IL-6 for 72 h, and the supernatants were measured for TIMP by sandwich EIA. Each bar shows the mean ± s.d. of six replicate experiments. The data are expressed as the amounts of TIMP produced by $1·5–2 \times 10^5$ cells in each well. $*P<0·05$; $**P<0·02$ *versus* the control by Student's *t*-test.

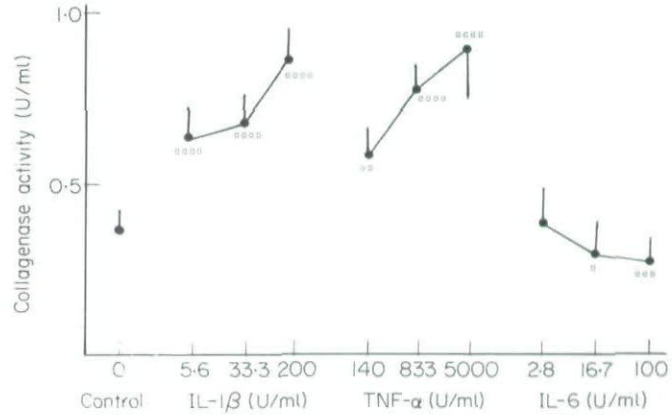

Fig. 2. The production of collagenase by chondrocytes and the effects of cytokines. Chondrocytes were cultured in Hanks' balanced slat solution (HBSS) for 72 h either with or without cytokine, and the supernatants were measured for collagenase activity. Each bar shows the mean ± s.d. of six replicate experiments. The data are expressed as the amount of collagenase activity produced by $1·5–2 \times 10^5$ cells in each well. $*P<0·05$; $**P<0·02$; $***P<0·01$; $****P<0·001$ *versus* the control by Student's *t*-test.

of TNF-α were added to cultures was significantly lower, at 1·58 ± 0·22, 1·33 ± 0·2, and 1·3 ± 0·13 ng/ml, respectively, compared with the control. On the other hand, IL-6 at concentrations of 2·8, 16·7 and 100 U/ml significantly enhanced TIMP production of the endothelial cells (Table 1).

TIMP production of synovial cells and the effects of cytokines

The TIMP level in the supernatant after 72 h of incubation of synovial cells without IL-1β (control) was 20·0 ± 2·3 ng/ml. TIMP production by synovial cells after the addition of 5·6, 33·3 and 200 U/ml of IL-1β was significantly higher compared with

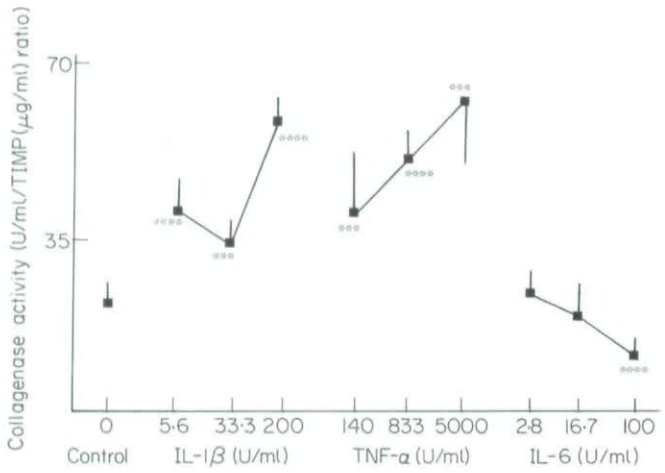

Fig. 3. The collagenase activity (U/ml)/TIMP (μg/ml) ratio in the supernatants of chondrocytes and the effects of cytokines. By using the data shown in Figs 1 and 2, the collagenase activity (U/ml)/TIMP (μg/ml) ratio was calculated as described in Materials and Methods. Each bar is the mean ± s.d. of six replicate experiments. $***P<0.01$; $****P<0.001$ *versus* the control by Student's *t*-test.

the control level. TIMP production was significantly lower than the control when 5000 U/ml of TNF-α were added to synovial cells. In addition, the synovial cell TIMP production in the presence of 2·8, 16·7 and 100 U/ml of IL-6 was significantly higher than the control (Table 2).

Collagenase/TIMP ratio in the supernatants of chondrocytes cultured with cytokines

When the effect of IL-1β on TIMP production during a 72-h culture was investigated, it was found that TIMP production was significantly higher in the presence of 33·3 U/ml of IL-1β compared with the control without IL-1β (17·2 ± 2·26 ng/ml). In addition, TIMP production in the presence of 5000 U/ml of TNF-α was significantly lower compared with that in the absence of TNF-α (17·4 ± 0·4 ng/ml). TIMP production by chondrocytes was significantly enhanced by the addition of 100 U/ml IL-6 (23·0 ± 3·4 ng/ml) compared with that without IL-6 (16·8 ± 2·6 ng/ml) (Fig. 1). Collagenase activity was also measured in the supernatants of chondrocytes cultured either with or without cytokines. IL-1β or TNF-α at any concentration enhanced collagenase production by chondrocytes, whereas IL-6 at 16·7 or 100 U/ml significantly inhibited collagenase production by chondrocytes (Fig. 2). Next, the collagenase activity (U/ml)/TIMP(μg/ml) ratio was calculated in order to study them for balance. The ratio was found to be significantly higher in the supernatants of chondrocytes cultured with IL-1β or TNF-α at any concentration studied. On the other hand, the ratio was significantly lower when the chondrocytes were cultured with 100 U/ml IL-6 (Fig. 3).

DISCUSSION

One of the most notable features of rheumatoid joints is the proliferation of the synovial membrane. The growth of synovial cells is often associated with the growth of the pannus, a vascular and fibrous extension of the perichondral portion of the synovial membrane, which grows over the cartilage and invades the cartilage matrix. The articular cartilage in RA is frequently overlaid by a cellular pannus that actively invades the cartilage [12]. The cartilage–pannus junction is infiltrated by mononuclear cells which release IL-β, TNF-α and IL-6. These processes are associated with uncontrolled endothelial cell migration and proliferation (vascularization). Synovial cells and chondrocytes are activated by IL-1β and TNF-α supposed to be derived from infiltrated macrophages or joint tissue cells themselves, such as proliferated synovial cells and chondrocytes. These activated cells produce enhanced amounts of MMP capable of degrading extracellular matrix components [8,13]. Thus, the eventual destruction of the rheumatoid joint is a consequence of synovial membrane proliferation and pannus invasion of the articular cartilage. Cultured endothelial cells also produce collagenase-like MMP and TIMP [14]. MMP, like collagenase, is required in the process of endothelial cell infiltration of the basement membrane in vascularization [15]. IL-6 is another cytokine produced by synovial cells, chondrocytes, endothelial cells and infiltrated macrophages. It has been shown that the effects of IL-6 on connective tissue cells are different from those of IL-1β. In the present study the effects of these cytokines on TIMP production by synovial cells, chondrocytes and endothelial cells were investigated.

The effects of cytokines on TIMP production by cultured cells were not consistent in our study. It appears that TIMP production in the cells differs from one type of cytokine to the other, and that the effect of the same cytokine on TIMP production also differs from cell to cell. IL-1β inhibited TIMP production in endothelial cells, but conversely increased TIMP production in synovial cells and chondrocytes. TNF-α inhibited TIMP production in endothelial cells, synovial cells and chondrocytes. IL-1β and TNF-α are known to enhance MMP production in endothelial cells, synovial cells and chondrocytes, as well as in human fibroblasts. This leads to the assumption that IL-1β enhances not only MMP production in synovial cells and chondrocytes, but enhances production of TIMP as well. The highest concentration of TNF-α used was 5000 U/ml. There were some non-adherent cells in each well after a 3-day culture, but there were no significant differences in the number of detached cells among each experimental condition for the synovial cells, chondrocytes and endothelial cells, suggesting that 5000 U/ml TNF-α was not cytotoxic. This implies that the inhibitory effect of TNF-α on TIMP production in these cell types was biologic.

IL-6 enhanced TIMP production in synovial cells, chondrocytes and endothelial cells, in contrast to TNF-α. It has been shown that IL-6 does not exhibit any effect on either synovial cell proliferation or MMP production in human fibroblasts [16]. In the present study, IL-6 at concentrations of 2·8, 16·7 and 100 U/ml significantly enhanced TIMP production in synovial cells and endothelial cells. These data suggest that IL-6 abolishes the MMP/TIMP imbalance, in contrast to the effects of IL-1β. Since the synovial cell pannus destroys the cartilage matrix by MMP-mediated mechanisms, the effects of IL-6 on MMP and TIMP production in synovial cells are assumed to be protective rather than destructive. In fact, the collagenase activity/TIMP ratio in chondrocytes cultured with IL-1β or TNF-α was significantly increased compared with the control, whereas the ratio was significantly lower than the control in chondrocytes cultured with IL-6. The results obtained suggest that IL-6 protects the cartilage from MMP-mediated degradation, in contrast to the destructive effects of IL-1β or TNF-α.

It has been suggested that normal synovial membranes produce TIMP and a little collagenase, whereas RA synovial membranes produce more collagenase, but only a little TIMP [17]. TIMP production by synovial cells is increased by the addition of steroid hormones, and collagenase production conversely decreases, while indomethacin has no effect on TIMP production by the synovial membrane of patients with RA and osteoarthritis (OA), although indomethacin raises TIMP production in normal synovial membranes [17]. If the rise in MMP exceeds the increase in TIMP in the synovial membranes and cartilaginous tissue, the destruction becomes more complete [18]. In the OA animal model and cartilage from OA patients, the same phenomenon has been illustrated [19,20]. When the collagenase and TIMP levels in the synovial fluid are compared between RA and OA, both show high levels in RA, and the collagenase/TIMP ratio is also significantly higher in RA [21].

The present observations demonstrate the important differences in the spectrum of biologic effects of IL-1β, TNF-α and IL-6 on connective tissue cells. IL-6 may thus serve as a protective mechanism that balances the catabolic effects of IL-1β and TNF-α. MMP play a key role in joint destruction in diseases such as RA and OA, in which the main pathological change is joint destruction. The balance between MMP and TIMP is therefore considered to be a key factor in both joint destruction and disease progression.

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