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Effect of heparinoid on the production of tissue inhibitor of metalloproteinases (TIMP)-3 in rheumatoid synovial fibroblasts

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Abstract

Heparinoid is one of the major contents of Mobilat widely used as an antirheumatic drug. To clarify the precise mechanisms of the antirheumatic effect of heparinoid, we investigated its effects on the production of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) from rheumatoid synovial fibroblasts stimulated (or not) with interleukin-1 alpha (IL-1α) at 100 units mL^{-1}. The expression of TIMP-3 mRNA was also investigated in a similar manner. The production of both MMPs and TIMPs and the expression of TIMP-3 mRNA were investigated by western-blot analysis and northern-blot hybridization, respectively. Under the stimulation of IL-1α, heparinoid increased the production of TIMP-3 in a concentration-dependent manner, but not TIMP-1, TIMP-2, MMP-1 or MMP-3. Heparinoid did not affect the expression of TIMP-3 mRNA that was increased by the stimulation of IL-1α. These findings suggest that the anti-rheumatoid effect of heparinoid may be due to increased production of TIMP-3. This increase in TIMP-3 may help redress the imbalance between the amounts of MMPs and TIMPs as observed in the joint tissues of rheumatoid arthritis and osteoarthritis patients.

Introduction

In patients with rheumatoid arthritis and osteoarthritis, proteolytic activity in the joint tissue is elevated, followed by the joint dysfunction. Matrix metalloproteinases (MMPs) are considered to be closely involved in the degradation of the extracellular matrix of articular cartilage in these arthritides. Several MMPs and their endogenous inhibitors (i.e., tissue inhibitors of metalloproteinases (TIMPs)) are usually up-regulated in rheumatoid arthritic and osteoarthritic joints (Nagase & Okada 1997). Because TIMPs generally inhibit the activity of MMPs by forming 1 : 1 molar complexes (Nagase & Okada 1997), the imbalance between the amount of MMPs and TIMPs become the primary pathogenic factor in generating the extent of extracellular matrix degradation as well as other pathologic changes in arthritic joints. Based on this rationale, the therapeutic strategy of inhibiting MMPs and elevating TIMPs activity in joint tissues seems to be a reasonable approach for preserving extracellular matrix integrity or modulating other pathologic events in both rheumatoid arthritis and osteoarthritis.

Interleukin-1 alpha (IL-1α) is an important pro-inflammatory cytokine involved in the pathogenesis of arthritis (McDonnell et al 1992; Arend & Dayer 1995), and induces rapid release of proteoglycan from chondrocytes (Yocum et al 1995). It has

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also been reported that IL-1α induced the production of MMP-1 and MMP-3 from rabbit cartilage explants (Saito et al 1997) and that IL-1 levels in synovial fluid were correlated with the progression of rheumatoid arthritis (Holt et al 1992; Kahle et al 1992).

One of the polysulfated glycosaminoglycans, heparinoid, is extracted from bovine tracheal and bronchial cartilage. Heparinoid exerts many biological actions in the same way as heparin, which has a similar chemical structure (Kido et al 2000). Although heparinoid is one of the major contents of Mobilat, widely used as an antirheumatic drug, the precise antirheumatic mechanism of heparinoid has not been fully clarified. However, intra-articular injection of Arteparon containing polysulfated glycosaminoglycans also normalized articular cartilage lesions induced by meniscectomy in the canine (Hannan et al 1987). Furthermore, it has been reported that pentosan polysulfate, which is a polysulfated polysaccharide like heparinoid, inhibited the cartilage degradation through the up-regulation of TIMPs activity in-vivo (Rogachefsky et al 1993), and increased the production of TIMP-3 in synovial fibroblasts of rheumatoid arthritis patients in-vitro (Takizawa et al 2000).

Therefore, we made an attempt to investigate the effect of heparinoid on the production of MMP-1, MMP-3, TIMP-1, TIMP-2 and TIMP-3 and also to investigate the expression of TIMP-3 mRNA in rheumatoid arthritis synovial fibroblasts by the stimulation of IL-1α. Furthermore, we compared the effect of heparinoid on the TIMP-3 production with the structurally similar agent, heparin, and the representative anti-inflammatory drug, indometacin.

Materials and Methods

Cell treatment

Rheumatoid arthritis synovial fibroblasts (Toyobo, Japan) were cultured in 25- or 75-cm^2 culture flasks (Becton Dickinson Labware, USA). When the cells reached confluence, the medium was changed to serum-free minimum essential medium (Gibco BRL, USA) containing 0.2% lactalbumin hydrolysate (Gibco BRL). Then, the cells were treated with heparinoid (Maruho, Japan) at 10,100 or 1000 μg mL^{-1}, heparin (Sigma, USA) at 1000 μg mL^{-1} or indometacin (Sigma) at 100 μM, in the absence or presence of IL-1α (Genzyme Techne, USA) at 100 units mL^{-1} for up to 5 days and the culture media were harvested. After harvesting the media, the cells were incubated for 30 min at 37°C in the phosphate-buffered saline (PBS; Sigma) containing 10 mM ethylenediaminetetraacetic acid (Dojindo Laboratories, Japan) to remove the cells remaining in the extracellular matrix. Then, extracellular matrix was lysed using sodium dodecyl sulfate (SDS) lysis buffer (500 μL; 50 mM Tris (pH 7.4), 0.5% SDS, 1 mM dithiothreitol) and then the lysates were sonicated before analysis.

Western blotting for MMPs and TIMPs

For western blotting, samples were collected from each 25-cm^2 culture flask of fibroblasts. The harvested media (200 μL) concentrated by precipitation with 10% trichloroacetate (Wako Pure Chemical, Japan) and extracellular matrix lysates (15 μL) were denatured by heating at 95°C for 5 min. The denatured samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (7.5% (for MMPs) or 12.5% (for TIMPs) total acrylamide; Bio-Rad, USA) under reduction. Then, the separated proteins were electroblotted onto polyvinylidene difluoride membrane (Bio-Rad).

After blocking non-specific binding with a blocking buffer (PBS containing 5% skimmed milk and 0.05% Tween 20) at room temperature overnight, the membranes were treated with mouse monoclonal antibodies specific to human MMP-1 (41-1E5) (Zhang et al 1993), MMP-3 (55-2A4) (Obata et al 1992), TIMP-1 (147-6D11) (Kodama et al 1990), TIMP-2 (67-4H11) (Fujimoto et al 1993) and TIMP-3 (136-13H4) (Farriss et al 1997) (Fuji Chemical Industries, Japan) at a 1/250, 1/4000, 1/250, 1/250 and 1/250 dilution, respectively for 2 h at room temperature. Subsequently, the membranes were treated with horseradish-peroxidase-linked anti-mouse IgG (Amersham Pharmacia Biotech) at a 1/4000 dilution for 2 h at room temperature. The immunoreactive bands were detected by X-ray films (Kodak, Japan) using ECL detection reagents (Amersham Pharmacia Biotech, UK). The blots on the X-ray films were scanned by GT-9000 (EPSON) and analysed by NIH image.

Preparation of probes for TIMP-3 and G3PDH

As described above, rheumatoid arthritis synovial fibroblasts were cultured and total RNAs were extracted by ISOGEN Reagent (Nippon Gene, Japan). The cDNAs complemented to total RNAs were synthesized using First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech). cDNAs were amplified with *Taq* DNA polymerase (Amersham Pharmacia Biotech) using specific primers for human TIMP-3 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA. The primers for TIMP-3 mRNA were complementary to

nucleotides 594–613 (5′-ACAGGTCGCGTCTATG-ATGG-3′) and 892–911 (5′-GTCTGTGGCATTGA-TGATGC-3′), whereas those for G3PDH mRNA corresponded to nucleotides 211–234 (5′-CCAC-CCATGGCAAATTCCATGGCA-3′) and 786–809 (5′-TCTAGACGGCAGGTCAGGTCCACC-3′). The polymerase chain reaction (PCR) products (DNAs) were used as templates to synthesize ^{32}P-labelled DNA probes using ready-to-go labelling kit (Amersham Pharmacia Biotech).

Northern-blot hybridization

For northern-blot hybridization, total RNA was extracted with ISOGEN Reagent from each 75-cm^2 culture flask of fibroblasts. The steady-state levels of each mRNA (i.e. the levels resulting from the cumulative effects of both mRNA transcription and degradation) were determined on northern blot. Equivalent amounts (10 μg) of total RNA were denatured by heating at 65°C for 15 min, dissolved in loading buffer containing ethidium bromide at 3.3 mg mL^{-1}, and were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde. The RNAs were blotted onto nitrocellulose membrane. The RNAs were cross-linked with membrane by UV exposure, and then hybridized overnight with the ^{32}P-labelled cDNA probe for human TIMP-3 and G3PDH synthesized as described above. After the non-specific hybrids were washed out, the membranes were exposed to X-ray films at −80°C overnight to visualize the specific blots sufficiently. The blots on the X-ray films were scanned by GT-9000.

Results

Effect of heparinoid on the production of MMP-1 and MMP-3

In the absence of IL-1α, the production of both MMP-1 and MMP-3 in the culture media were not detectable (Figure 1, lane 1), and were not affected by the treatment with heparinoid at 1000 μg mL^{-1} (Figure 1, lane 2). When stimulated with IL-1α, the production of MMP-3 was markedly increased compared with non-treated cells despite MMP-1 being undetectable (Figure 1, lane 3). The detected MMP-3 was only the latent form (57 kDa band), but not the active form (45 kDa band). The addition of heparinoid at 1000 μg mL^{-1} into these IL-1α-treated cultures did not enhance the production of either MMP-1 or MMP-3 (Figure 1, lane 4). In addition, heparinoid at 10 and 100 μg mL^{-1} did not affect the production of MMP-1 or MMP-3, either in the presence or absence of IL-1α. Heparin and indometacin also had no effect on production (data not shown).

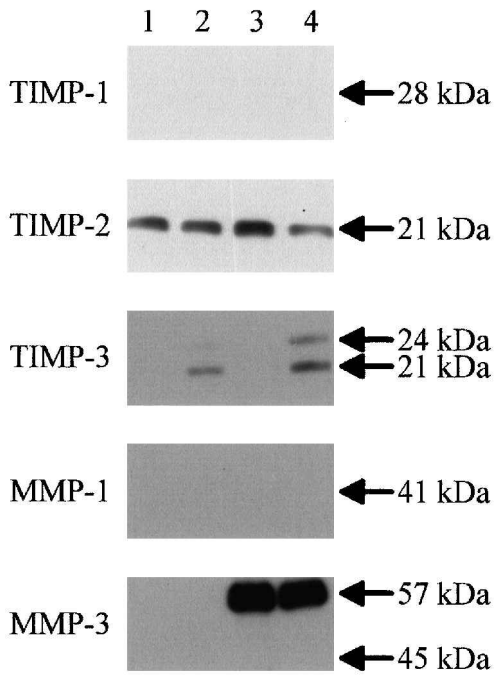

Figure 1 Western-blot analysis of the effect of heparinoid on the production of tissue inhibitors of metalloproteinases (TIMPs) and matrix metalloproteinases (MMPs) by rheumatoid arthritis synovial fibroblasts. Lane 1, non-treated; lane 2, heparinoid at 1000 μg mL^{-1} alone; lane 3, IL-1α at 100 units mL^{-1} alone; lane 4, heparinoid at 1000 μg mL^{-1} in the presence of IL-1α at 100 units mL^{-1}. Data indicate a representative example of two separate experiments.

Effect of heparinoid on the production of TIMP-1, TIMP-2 and TIMP-3

The basal production of TIMP-2 in the culture media was detectable, but not TIMP-1 and TIMP-3 (Figure 1, lane 1). In the absence of IL-1α, the treatment with heparinoid at 1000 μg mL^{-1} induced the production of 21 kDa of TIMP-3, but failed to alter the basal production of TIMP-1 and TIMP-2 (Figure 1, lane 2). The production levels of TIMP-1, TIMP-2 and TIMP-3 stimulated with IL-1α alone were similar to those of non-treated cells (Figure 1, lane 3). The additional treatment with heparinoid markedly enhanced the production of TIMP-3 compared with the cells treated with heparinoid alone and a part of TIMP-3 products were shifted from 21 kDa to 24 kDa by *N*-glycosylation (Apte

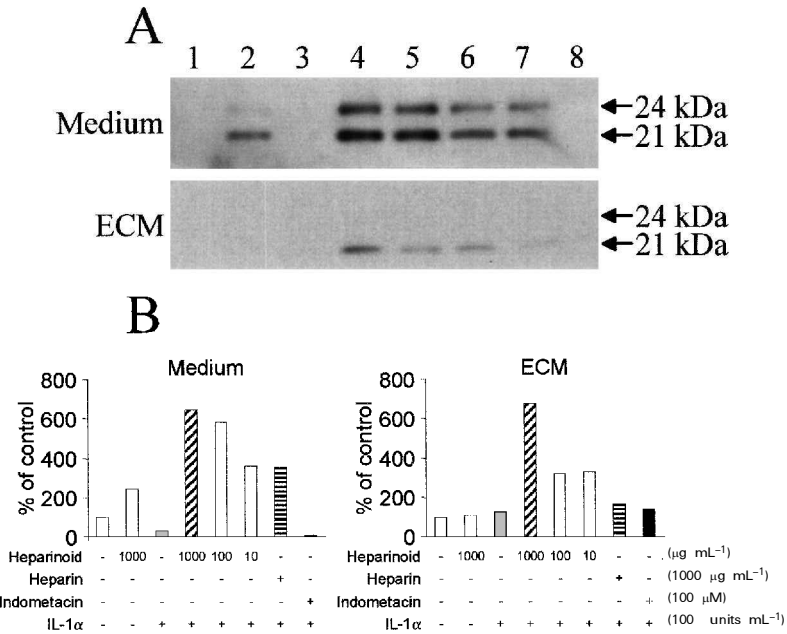

Figure 2 A. Western-blot analysis of the effect of heparinoid on the production of tissue inhibitors of metalloproteinases (TIMP)-3 by rheumatoid arthritis synovial fibroblasts (in the culture media and extracellular matrix (ECM)) in the presence of IL-1α. The production level of TIMP-3 induced by heparinoid was compared with that induced by heparin or indometacin treatment. Lane 1, non-treated; lane 2, heparinoid at 1000 μg mL^{-1} alone; lane 3, IL-1α at 100 units mL^{-1} alone; lanes 4–6, heparinoid at 1000, 100 or 10 μg mL^{-1} in the presence of IL-1α at 100 units mL^{-1}; lane 7, heparin at 1000 μg mL^{-1} or lane 8, indometacin at 100 μM in the presence of IL-1α at 100 units mL^{-1}. Data indicate a representative example of two separate experiments. B. Quantitative analysis of TIMP-3 production was performed using NIH image. Data indicate the mean of two separate experiments.

et al 1995). However, TIMP-1 and TIMP-2 production was not affected by this treatment (Figure 1, lane 4).

When the cells were non-stimulated or stimulated by IL-1α alone, TIMP-3 in both the culture media and extracellular matrix lysates was undetectable (Figure 2A, lanes 1 and 3). The slight induction of TIMP-3 synthesis was observed as two different products in the culture media treated with heparinoid alone (the upper panel in Figure 2A, lane 2) but not in the extracellular matrix lysates (the lower panel in Figure 2A, lane 2). Furthermore, TIMP-3 in the culture media and the extracellular matrix lysates was increased in a concentration-dependent manner by treatment with heparinoid (10–1000 μg mL^{-1}) in the presence of IL-1α (Figure 2A, lanes 4–6). Following the treatment with heparinoid at 1000 μg mL^{-1}, TIMP-3 production was increased 2.6- and 6.1-fold by the stimulation with IL-1α in the culture media (Figure 2B, left) and the extracellular matrix (Figure 2B, right), respectively. TIMP-3 was observed as double bands of 21 and 24 kDa in the culture media and observed as a single band of 21 kDa in the extracellular matrix lysates. The production level of TIMP-3 in the culture media induced by heparin at 1000 μg mL^{-1} (the upper panel in Figure 2A, lane 7) corresponded to that of heparinoid at 10 μg mL^{-1} (the upper panel in Fig 2A, lane 6) in the presence of IL-1α, but not detected in the extracellular matrix lysates (the lower panel in Figure 2A, lane 6). In addition, indometacin did not provoke the production of TIMP-3 synthesis at a concentration of 100 μM in either the culture media or the extracellular matrix lysates (Figure 2A, lane 8).

Furthermore, we examined the effect of heparinoid with the various treatment periods on the TIMP-3 synthesis in both culture media and extracellular matrix lysates in the presence of IL-1α. When the cells were not treated with heparinoid, the production of TIMP-3 was undetectable in both the culture media and the extracellular matrix lysates from days 1–5 (Figure 3, lanes 1–5). On the other hand, treatment with heparinoid led to the accumulation of TIMP-3 in the culture media from day 1 up to day 5 (the upper panel in Figure 3, lanes 6–10), the marked increase was especially observed between days 4 and 5. The existence of TIMP-3 was observed as a single band of 21 kDa from days 1 to 5 in the extracellular matrix lysates (the lower panel in Figure 3, lanes 6–10), and did not increase during these periods.

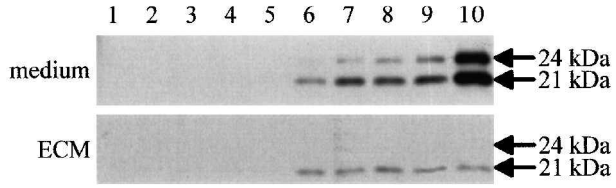

Figure 3 Western-blot analysis of the effect of heparinoid on the production of tissue inhibitors of metalloproteinases (TIMP)-3 by rheumatoid arthritis synovial fibroblasts (in the culture media and extracellular matrix (ECM)). The cells were non-treated (lanes 1–5) or treated (lanes 6–10) with heparinoid at 1000 μg mL^{-1} in the presence of IL-1α at 100 units mL^{-1} for 1–5 days. Each lane of 1–5 and 6–10 corresponds to the treatment period of 1–5 days. Data indicate a representative example of two separate experiments.

Effect of heparinoid on the expression of TIMP-3 mRNA

TIMP-3 mRNA expression was investigated and estimated by comparison with homoeostatic expression of G3PDH mRNA. By northern-blot hybridization, three different transcripts of 5.0, 2.6 and 2.4 kbp were detectable in non-stimulated rheumatoid arthritis synovial fibroblasts as the basal expression of TIMP-3 mRNA (Figure 4A, lane 1). Heparinoid did not affect the basal expression (Figure 4A, lane 2). The expression was increased by stimulation with IL-1α (Figure 4A, lane 3) and additional treatment with heparinoid (Figure 4A, lanes 4–6) or heparin (lane 7) did not induce further expression.

The transcription level of TIMP-3 reached a plateau on day 1, which was maintained through to day 5 in the presence of IL-1α (Figure 4B, lanes 1–3) and no effect was observed by the additional treatment of heparinoid (Figure 4B, lanes 4–6).

Additionally, G3PDH mRNA was not affected by stimulation with IL-1α or by treatment with heparinoid or heparin (Figures 4A and 4B).

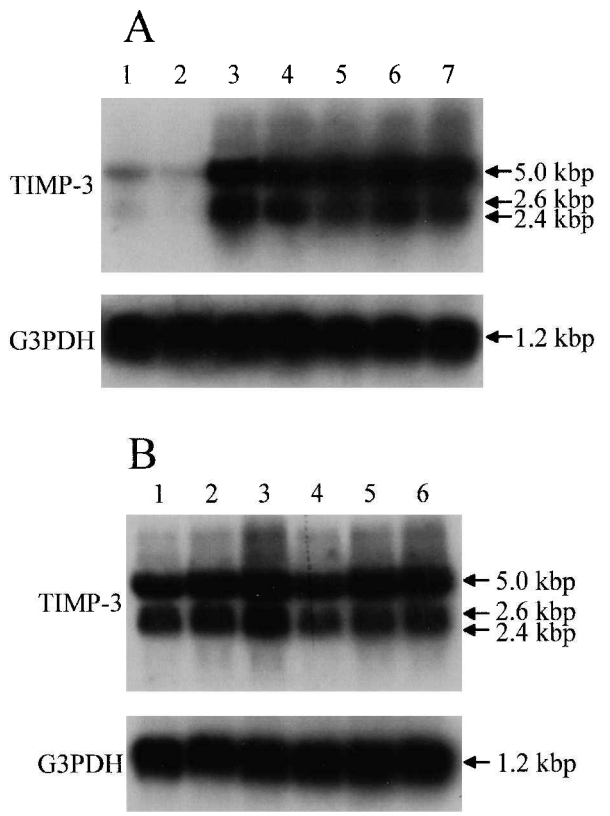

Figure 4 Northern-blot hybridization analysis of effect of heparinoid on the expression of tissue inhibitors of metalloproteinases (TIMP)-3 mRNA by rheumatoid arthritis synovial fibroblasts. A. Rheumatoid synovial fibroblasts were treated with heparinoid at 10–1000 μg mL^{-1} or heparin at 1000 μg mL^{-1} in the absence (lanes 1, 2) or presence (lanes 3–7) of IL-1α at 100 units mL^{-1} for 5 days. Lane 1, non-treated; lane 2, heparinoid at 1000 μg mL^{-1} alone; lane 3, IL-1α at 100 units mL^{-1} alone; lanes 4–6 and lane 7, heparinoid at 1000–10 μg mL^{-1} and heparin at 1000 μg mL^{-1} in the presence of IL-1α at 100 units mL^{-1}, respectively. B. Rheumatoid synovial fibroblasts were treated with heparinoid at 1000 μg mL^{-1} in the presence of IL-1α at 100 units mL^{-1} for 1 (lanes 1, 4), 3 (lanes 2, 5) or 5 (lanes 3, 6) days. Lanes 1–3 and lanes 4–6 show cells treated with IL-1α and IL-1α plus heparinoid, respectively. Data indicate a representative example of two separate experiments.

Discussion

Since MMPs are mainly involved in cartilage degradation in arthritis, TIMPs attenuating the activity of MMPs have the potential to act as endogenous anti-arthritic substances (Nagase & Okada 1997). Because IL-1α is known to produce MMPs, IL-1α may be important for the pathogenesis related to MMPs (McDonnell et al 1992; Arend & Dayer 1995). Heparinoid selectively increased the production of TIMP-3 in a concentration-dependent manner under stimulation with IL-1α without showing effects on the production of TIMP-1, TIMP-2, MMP-1 and MMP-3. Although heparin also had a similar effect, this effect was 100-fold weaker than heparinoid. Indometacin had no effect on the production of TIMPs. Heparinoid is thus a more selective and stronger enhancer of TIMP-3 production than either heparin or indometacin.

Cytokines and growth factors modulating the cellular production of MMPs and TIMPs commonly act at their transcription levels (Nagase & Okada 1997). Therefore, we examined the effect of heparinoid on the gene expression of TIMP-3 in the synovial fibroblasts by northern-blot hybridization. Although IL-1α enhanced

TIMP-3 mRNA expression, heparinoid did not affect this expression in the presence or absence of IL-1α. Additionally, pentosan polysulfate is also considered to regulate the production of TIMP-3 at post-transcription level (Takizawa et al 2000). These findings suggest that heparinoid enhances the production of TIMP-3 at post-transcription level. Interestingly, heparinoid had a greater effect on the production of TIMP-3 under IL-1α stimulation compared with the absence of IL-1α. Thus, the enhancement of TIMP-3 gene expression may play an important role in the exertion of the action of heparinoid.

Recent studies have revealed that TIMP-3 has other important biological activity apart from its function as an MMP inhibitor. TIMP-3 selectively inhibits the activity of tumour necrosis factor (TNF)-α-converting enzyme (Amour et al 1998). TNF-α, one of the important pro-inflammatory cytokines, is produced mainly by the lining cells of the synovial membrane in rheumatoid arthritis (Chu et al 1991). Thus, TNF-α production from its precursor on the cell membranes may be suppressed when TIMP-3 is increased in the rheumatoid arthritis synovial cells by treatment with heparinoid. Another important effect of TIMP-3 is to suppress endothelial cell proliferation induced by basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF) (Anand-Apte et al 1997). It is considered that angiogenesis observed in rheumatoid arthritis synovium is particularly induced by bFGF and VEGF (Koch 1998). The proliferation of human umbilical vein endothelial cells induced by bFGF was inhibited by heparinoid (Kido et al 2000). Therefore, heparinoid enhancing TIMP-3 production from the synovium may inhibit angiogenesis induced by these growth factors in the pannus.

In summary, heparinoid selectively increased the production of TIMP-3 from rheumatoid arthritis synovial fibroblasts in-vitro without affecting production of MMP-1, MMP-3, TIMP-1 and TIMP-2. This action of heparinoid seemed to be regulated at the post-transcription level and was more marked in the presence of IL-1α. From these findings, one of the mechanisms of the antirheumatic effect of heparinoid may be via the selective enhancing effect on the production of TIMP-3, leading to improvement in the imbalance of MMPs.

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