Mod Rheumatol (2002) 12:160–166

ORIGINAL ARTICLE

Tohgo Nonaka · Hiraku Kikuchi · Satoshi Sohen
Kanji Fukuda · Chiaki Hamanishi · Seisuke Tanaka

Comparison of the inhibitory effects of two types (90 kDa and 190 kDa) of hyaluronic acid on the expression of fibrinolytic factors in human synovial fibroblasts

Received: August 17, 2001 / Accepted: December 13, 2001

Abstract Hyaluronic acid (HA) has been shown to be clinically effective, and is currently used for the treatment of arthropathy. We previously reported that HA of molecular weight 90 kDa (90-HA) inhibits the fibrinolytic factors in human synovial fibroblasts. In the present study, we investigated the effect of high molecular weight (190 kDa) HA (190-HA) compared with 90-HA on the pericellular fibrinolytic system of human synovial fibroblasts in osteoarthritis (OA) and rheumatoid arthritis (RA). Human synovial fibroblasts were obtained from synovial tissues of OA and RA, and were cultured in the presence and absence of 90-HA and 190-HA. Antigens of urokinase-type plasminogen activator (u-PA) and PA inhibitor-1 (PAI-1) were measured by ELISA, and the u-PA activity of the cell surface fraction was evaluated by electrophoretic enzymography. The binding assay of u-PA and the immunocytochemical analysis of u-PA were performed to detect u-PA receptor (u-PAR). HA inhibited the secretion of both u-PA and PAI-1 antigens from the synovial fibroblasts of OA to their conditioned medium, and the degree of inhibition was more effective in OA than in RA. The u-RA binding assay to these cells showed that both 90-HA and 190-HA slightly decreased the maximal number of binding sites (Bmax) in OA. However, in RA, stimulation with 90-HA and 190-HA decreased Bmax by a half and a quarter, respectively. Immunohistochemical analysis showed that u-PAR was constitutively expressed in both synovial fibroblasts, but if these cells were treated with HA, the decrease in the staining of u-PAR was more pronounced in RA than in OA. Furthermore, the degree was more effective with 190-HA than with 90-HA. HA inhibited the pericellular fibrinolytic activity mediated by the u-PA/u-PAR system in synovial fibroblasts of OA and RA, and 190-HA inhibited it more effectively than 90-HA.

T. Nonaka (✉) · H. Kikuchi · S. Sohen · K. Fukuda · C. Hamanishi · S. Tanaka
Department of Orthopaedic Surgery, Kinki University School of Medicine, 377-2 Ohnohigashi, Osakasayama 589-8511, Japan
Tel. +81-72-366-0221 (ext. 3212); Fax +81-72-366-0206
e-mail: cra@med.kindai.ac.jp

Key words Hyaluronic acid (HA) · Rheumatoid arthritis (RA) · Synovial fibroblasts · Urokinase-type plasminogen activator receptor (u-PAR)

Introduction

Several proteases are involved in the inflammation of rheumatoid arthritis (RA), osteoarthritis (OA), and other joint disorders. Two types of plasminogen activators (PA),[1] tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), which both belong to the serine protease family, activate the zymogen plasminogen to its active form, plasmin.[2] t-PA plays a major role in intravascular fibrinolysis because of its high affinity for fibrin, and the enhancement of enzymatic activity in the presence of fibrin or fibrin degradation products. Recent studies have shown that t-PA activity can be modified by binding to its specific receptor (t-PAR) expressed on the surface of endothelial cells.[3] On the other hand, u-PA is predominantly involved in the extravascular fibrinolysis that occurs in cell migration, tissue remodeling, and the cell invasion and metastasis of cancer cells.[4] u-PA is also associated with mineralized tissues,[5] and appears to be involved in the degradation of connective tissue.[6] Thus, u-PA-dependent proteolysis is implicated in turnover of tissues under physiological and pathological conditions. However, several PA inhibitors (PAI) exist that inactivate PA activity. The most physiological and pathological PAI is type 1 PA inhibitor (PAI-1),[7] which binds to and limits the activity of both t-PA and u-PA. In addition to these fibrinolytic factors, a cellular receptor specific for u-PA (u-PAR) has been demonstrated in many cell types, and the molecular and biological functions of u-PAR have now been identified in detail.[8–10] u-PA is primarily secreted from the cells in a single-chain form (scu-PA) which possesses little or no enzymatic activity. Since scu-PA binds to u-PAR-expressing cells, and the scu-PA-bound u-PAR can be converted to the active two-chain form (tcu-PA) remaining on u-PAR,[11] u-PA activity can be exhibited on the surface of cells, thus localizing its enzymatic activity. This condensed u-PA activ-

ity from u-PAR on the cell surface is closely associated with many biological events. Therefore, the level of fibrinolysis in plasma and in synovial fluid may be reflected by the balance between PA and PAI, as well as the expression of t-PAR and/or u-PAR. In fact, PA and PAI play important roles in the onset and progression of arthropathy.[12] In addition, we have previously demonstrated that u-PA and u-PAR, as well as other fibrinolytic components, are regulated in human osteoblast-like and osteosarcoma cell lines,[13,14] indicating that these factors were involved in the bone resorption process.

The main source of intraarticular fibrinolytic factors (u-PA, t-PA, and PAI-1) is synovium, but cartilage only produces small amounts. Thus, the fibrinolytic system is mainly modulated by synovium rather than by cartilage in the joints of OA and RA patients. The uncontrolled enhancement of proteolytic activity in synovial tissues may be one of the pathological features of OA and RA, since synovial fibroblasts are activated by the stimulation of inflammatory cytokines such as interleukin 1, interleukin 6, or prostaglandin E_2.[15–18] Some of these cytokines preferably stimulate the expression of fibrinolytic factors in synovium,[19,20] and undesirable matrix destruction would be triggered by this fibrinolytic activity. Thus, the difference between OA and RA may be attributed to the degree of tissue degradation caused by these proteolytic factors. In fact, the synovial tissues of RA patients expressed u-PA, t-PA, and PAI-1 mRNAs more frequently than those of OA patients.[21] In addition, there are more u-PAR-positive synovial fibroblasts in RA than in OA.[22] Furthermore, endothelial cells from OA and RA patients possessed more u-PAR than those from normal controls.

Therefore, one target of OA and RA therapy should be the down-regulation of the excessive fibrinolytic activity that may cause pathological destruction in synovium.

Hyaluronic acid (HA) is one practical agent used in the therapy of OA and RA. Two types of HA are now available: one with a molecular weight of 90 kDa (90-HA) derived from cockscomb, and one with a molecular weight of 190 kDa (190-HA) produced by *Streptococcus equi*. When HA is introduced into the extracellular matrix (ECM), it acts to protect the injured ECM and to reduce friction.[23] HA is also known specifically to increase the viscosity of synovial fluid in the joints of OA patients.[24] Furthermore, HA has been shown to inhibit inflammation and restore cartilage in a dose-dependent manner in vitro.[25] We have previously reported that 90-HA inhibited the expression of u-PA, PAI-1, and u-PAR in human synovial fibroblasts of OA and RA.[26] We found that the administration of 90-kDa HA (90-HA) (25 mg) to patients with OA of the knee improved in their clinical parameters, and that this improvement was found to be associated with a gradual decrease in u-PA activity in their synovial fluid about 2–4 weeks after the 90-HA treatment.[27]

In this study we compare the effect of 90-HA with that of high molecular weight 190-HA on the secretion of fibrinolytic factors, u-PA, and PAI-1, as well as on the expression of u-PAR in synovial fibroblasts derived from OA and RA tissues.

Materials and methods

Materials

The following materials were purchased from the sources indicated: diisopropyl fluorophosphate (DFP) (Sigma, St. Louis, MO, USA); phosphatidylinositol-specific phospholipase C (PI-PLC) (Boehringer Mannheim, Germany); MW 90 kDa hyaluronic acid (Kaken Pharmaceuticals, Tokyo, Japan); MW 190 kDa hyaluronic acid (Chugai Pharmaceuticals, Tokyo, Japan); Eagle's MEM (Nissui Seiyaku, Tokyo, Japan); fetal calf serum (FCS) (GIBCO, Grand Island, NY, USA); bovine thrombin (Mochida Pharmaceuticals, Tokyo, Japan); plasminogen containing bovine fibrinogen (Organon Technika, Boxtel, The Netherlands); iodogen (Pierce Chemical Co., Rockford, IL, USA); Na-^{125}I (Amersham International, Amersham, UK). For immunocytochemistry, an anti-u-PAR was obtained from American Diagnostica (Greenwich, CT, USA). The purified high molecular weight two-chain urokinase-type plasminogen activator was a gift from Dr. Nobuhara (Mochida Pharmaceuticals, Tokyo, Japan). Antibodies against u-PA or t-PA were raised in rabbits, as described elsewhere.[28] Monoclonal antibody against u-PAR was a gift from Prof. Danø (Finsen Laboratory, Copenhagen, Denmark). All other reagents and chemicals were of the highest grade available.

Cell culture

The human synovial fibroblasts were isolated by the explant method from synovial tissues from OA ($n = 5$) and RA ($n = 5$) patients undergoing total knee arthroplasty.[29] OA patients were diagnosed by clinical and radiological criteria,[30] and RA patients fulfilled the American College of Rheumatology (ACR) criteria.[31] Fibroblasts were observed to migrate from the synovium in the primary culture. These migrating cells were selectively isolated and subcultured until the cell populations morphologically and homogeneously became fibroblasts by using 0.125% trypsin and 0.5 mM ethylenediaminetetraacetic acid in calcium- and magnesium-free phosphate-buffered saline (PBS). They were maintained in a 25-cm^2 flask in Eagle's MEM containing 10% FCS at 37°C under 5% CO_2. Fibroblasts thus selected after 5–8 passages were used for the experiments. These fibroblasts were seeded in a 24-well multiplate at about 2.2×10^5 cells/well, and they filled the sheet within 12 h with no proliferation, since the number of cells was enough to cover the bottom of each well. Both the number and the viability of cells were unchanged 48 h after seeding, thus maintaining 2.2×10^5 cells/well and over 95% viability after trypan blue staining after tripsinization.

Stimulation of synovial fibroblasts with HA

Confluent synovial fibroblasts (2.2×10^5 cells/well in 24 wells) were washed with PBS and added to FCS-free MEM. The cells were incubated for 48 h at 37°C in the presence of

either 90-HA or 190-HA (1 mg/ml). Antigens of u-PA and PAI-1 in the conditioned medium were measured by their corresponding ELISAs, and u-PA activities recovered from the cell surface were measured by electrophoretic enzymography.

PI-PLC treatment of synovial fibroblasts

Confluent synovial fibroblasts were incubated in FCS-free MEM for 48 h in the presence and absence of two classes of HA. After harvesting the conditioned medium, the cells were washed briefly with PBS and treated with PI-PLC (0.2 U/ml) for 1 h at 37°C. The u-PA activities in the conditioned medium and in the fraction obtained by the treatment with PI-PLC (cell-surface fraction) were analyzed. The u-PA activity of the cell surface fraction reflects the u-PA activity associated with u-PAR.

Measurements of PA activity in the cell-surface fraction

Cell-surface fractions were obtained by PI-PLC treatment of the confluent cells (2.2×10^5 cells/well) in FCS-free MEM. The PA activities and their molecular mass weights were simultaneously analyzed by electrophoretic enzymography[32] as follows. For the separation gel, 2 ml acrylamide (30 g/100 ml) and bisacrylamide (1 g/100 ml), 240 µl bovine plasminogen-rich fibrinogen (1.5 mg/ml) and 1.5 ml 1.5 M Tris-HCl buffer (pH 8.8) were mixed. Then, 24 µl thrombin (10 NIH U/ml), 60 µl of 10% sodium dodecyl sulfate, 10 µl of N, N, N, N′-tetramethylethylenediamine, and 60 µl of 10% ammonium peroxodisulfate were added. The regular method was used for the preparation of a stacking gel. In order to identify the PAs in the cell-surface fractions immunologically, each sample was treated with either anti-u-PA antibody (1 mg/ml) or anti-t-PA antibody (1 mg/ml) at 37°C for 2 h before electrophoresis. After electrophoresis, the gel was soaked in 2.5% Triton X-100 for 2 h, and then incubated in 0.1 M glycine buffer (pH 8.3) at 37°C for 24–36 h. After soaking in 50% trichloroacetic acid solution for 1 h, and then in 7% ethanol for 1 h, the gel was stained with Coomassie brilliant blue. After destaining with 7% acetic acid, the gel developed a lysed zone if PA was present.

Measurements of antigens of u-PA and PAI-1

The amounts of u-PA and PAI-1 antigens in the conditioned medium were determined by a u-PA ELISA kit (Monozyme, Virum) and a PAI-1 ELISA kit (Technoclone, Vienna, Austria).

Binding assay of u-PA to synovial fibroblasts

The purified high molecular weight tcu-PA was pretreated with DFP, which blocks its enzymatic activity, and radiolabeled using iodogen and Na-^{125}I (referred to below as ^{125}I-DFP-u-PA). Since the ^{125}I-DFP-u-PA does not interact with PAI-1, which may be preferably accumulated in the ECM of fibroblasts, the binding of this ligand will be an authentic interaction with u-PAR expressed on the cells. Synovial fibroblasts in a 24-well multiplate were incubated with 1 mg/ml HAs at 37°C for 48 h. Then the intrinsic u-PA which had bound to the cell surface was dissociated by acid treatment, as follows. After harvesting the conditioned medium, u-PA bound to the cell surface was recovered by incubating the cells with 50 mM glycine/HCl (pH 3.0) for 3 min at room temperature. Thereafter, 100 mM Tris/HCl (pH 8.0) was added to restore the pH to 7.4.[33] After removing the cell-surface fraction, the cells were rinsed with PBS. The cells were then incubated with ^{125}I-DFP-u-PA (0–15 nM), with or without on excess amount of unlabeled u-PA (3000 nM), at 4°C for 90 min in a 24-well multiplate. The radioactivity recovered from the cell lysate, which was lysed with 0.5% Triton X-100, was measured with a multi-crystal gamma counter LB-2100 (Berthold Wildbad). The binding characteristics of u-PA to the cells were analyzed by Scatchard plots to evaluate the maximal number of binding sites (Bmax) and dissociation constants (Kd).[34]

Immunocytochemistry

Cells were incubated for 48 h at 37°C with 1 mg/ml HAs. They were then fixed in Zambony's solution for 8 h at room temperature. After washing with PBS, fixed samples were incubated for 12 h in a humidified chamber, and monoclonal antibody against u-PAR was reacted at 4°C. The monoclonal antibody was diluted (1:50) with PBS containing 0.25% carrageenan, 2% normal horse serum, and 0.5% Triton X-100. The samples were washed with PBS and then incubated with a biotinylated anti-mouse IgG (1:200) (vector) for 2 h. After washing again with PBS, the samples were dipped in 3 mM sodium metaperiodate solution for 1 h to block the endogenous peroxidase activity. After washing, avidin:biotinylated horseradish peroxidase complex dissolved in PBS containing 0.5% Triton-X 100 was reacted on the samples for 1 h. The final reaction was done by incubating the samples with 0.003% hydrogen peroxide and 0.17 mg/ml diaminobenzidine tetrahydrochloride in 0.05 M Tris-HCl buffer (pH 7.4) for 3–5 min.[35] For the confocal microscopy, FITC-conjugated anti-mouse IgG (1:200) was used. The cells were analyzed under a laser scanning confocal microscope (MRC 2400 LSX imaging system, Bio-Rad, UK) equipped with an argon laser adjusted to an output of 250 mW at 488 nm excitation and 525 nm emission.

Results

Effect of HA on the secretion of u-PA and PAI-1 antigens

To clarify the effect of two types of HA on the secretion of u-PA and PAI-1 antigens, synovial fibroblasts were incubated with 1 mg/ml HAs for 48 h at 37°C. These two kinds of HA on their own did not have any effects on either the number or the viability of cells during this period. As shown

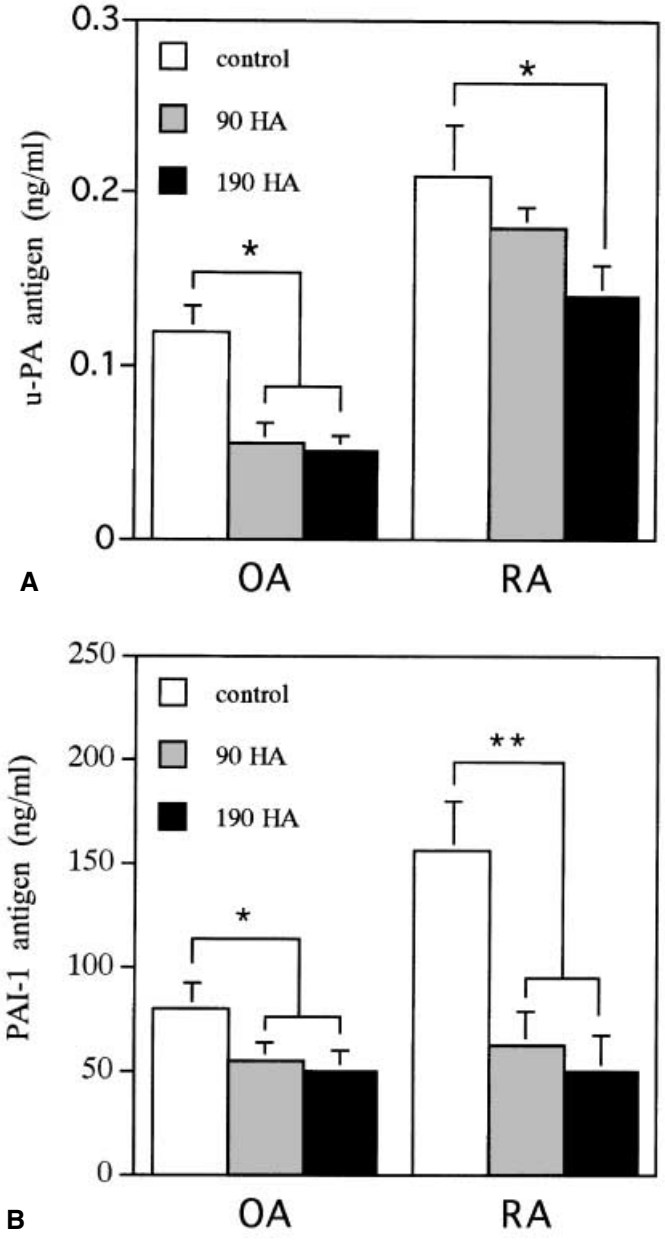

Fig. 1. Secretion of urokinase-type plasminogen activator (*u-PA*) **(A)** and PA inhibitor-1 (*PAI-1*) **(B)** antigens in a conditioned medium of synovial fibroblasts of osteoarthritis (*OA*) or rheumatoid arthritis (*RA*) stimulated with either 90 kDa hyaluronic acid (*90-HA*) or 190 kDa hyaluronic acid (*190-HA*). The cells (2.2×10^5 cells/well) were cultured in MEM without fetal calf serum (FCS) for 48 h at 37°C in the presence or absence of 1 mg/ml HA. Values are expressed as means ± standard deviation of five independent experiments. $*P < 0.01$; $**P < 0.05$

in Fig. 1A, both HAs significantly reduced the secretion of u-PA antigen into the conditioned medium of synovial fibroblasts of OA. However, there was no significant difference in u-PA antigen between the treatments with 190-HA and with 90-HA. In contrast, the treatment of synovial fibroblasts of RA with 90-HA showed a tendency to decrease the u-PA secretion, but the changes were not statistically significant. On the other hand, 190-HA significantly reduced the u-PA secretion from cells of RA.

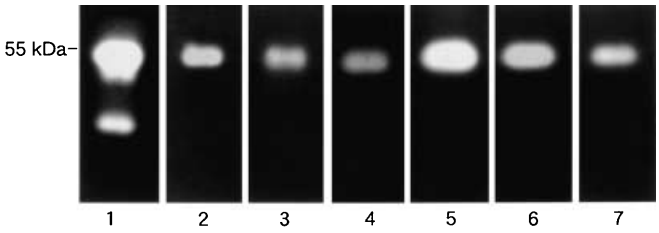

Fig. 2. The u-PA activity in the cell-surface fraction of synovial fibroblasts of OA (lanes 2, 3, and 4) and RA (lanes 5, 6, and 7) with or without (lanes 2 and 5) treatment with 90-HA (lanes 3 and 6) and 190-HA (lanes 4 and 7). After the cells were incubated in MEM without FCS for 48 h at 37°C in the presence or absence of HAs, the cell-surface fractions were obtained by PI-PLC treatment. The samples were analyzed by electrophoretic enzymography. Lane 1 shows the u-PA standard

The secretion of PAI-1 antigen was significantly reduced in synovial fibroblasts of both OA and RA treated with the same dose of two classes of HA for 48 h (Fig. 1B). It is notable that the suppression of PAI-1 secretion by the two HAs was stronger in cells of RA than in those of OA. However, there was no significant difference in the suppressive effects of PAI-1 secretion between treatments with 190-HA or 90-HA in any cell of both OA and RA.

Effect of HA on u-PA activity in the cell-surface fraction

In order to evaluate the amount of intrinsic u-PA that is binding to u-PAR on the cell surface, the u-PA activity in the cell-surface fraction was measured by electrophoretic enzymography (Fig. 2). The u-PA activity at a molecular weight of 55 kDa, corresponding to a high molecular weight u-PA, was detected in each sample of OA and RA, but the band of 33 kDa corresponding to a low molecular weight u-PA was not detectable in every sample (lanes 2–7). To confirm that the 55 kDa band originated from u-PA, the immunological study was performed by using anti-u-PA antibody and anti-t-PA antibody. The 55-kDa lysis band did not appear at all if the sample had been treated with anti-u-PA antibody before electrophoresis. On the other hand, the lysis band was not changed by treatment with anti-t-PA antibody. Thus, it is concluded that the 55-kDa molecule was immunologically identified as u-PA and not t-PA. HA treatment slightly decreased the 55-kDa activity in OA synovial fibroblasts, but no significant change was observed between the samples treated with 90-HA or 190-HA (lanes 3 and 4). In RA, both HAs decreased u-PA activity in the cell-surface fraction, and this effect was more pronounced with 190-HA than that with 90-HA (lanes 5–7).

Binding of ^{125}I-u-PA to synovial fibroblasts

The binding assay of u-PA to synovial fibroblasts of OA and RA was performed using ^{125}I-DFP-u-PA as a ligand. The u-PA-specific binding curve (data not shown) was obtained by subtracting the nonspecific binding (15%–25%) from the total binding in cells of both OA and RA. The binding

Table 1. Binding parameters of ^{125}I-DFP-u-PA to the synovial fibroblasts of OA and RA with or without treatment with 90-HA or 190-HA

		Kd (nM)	Bmax (10^4 binding sites/cell)
OA	Control	27.2 ± 6.9	2.82 ± 0.12
	90-HA	23.3 ± 3.2	2.30 ± 0.18*
	190-HA	26.8 ± 6.1	2.16 ± 0.20*
RA	Control	20.5 ± 4.1	10.26 ± 0.28
	90-HA	16.1 ± 2.7	6.22 ± 0.33**
	190-HA	14.3 ± 2.2	2.55 ± 0.11**

^{125}I-u-PA (0–15 nM) was added to acid-treated synovial fibroblasts which had been incubated with either 90-HA (1 mg/ml) or 190-HA (1 mg/ml) for 48 h. Excess amounts of unlabeled u-PA (3000 nM) were simultaneously added to the cells to measure the nonspecific binding. These binding parameters were obtained by Scatchard analysis. Values are expressed as means ± standard deviation from four independent experiments.
*$P < 0.05$; **$P < 0.01$

feature was characterized by Scatchard analysis. The binding parameters are shown in Table 1. The Kd values of OA cells were a little higher than those of RA cells, but they were not significantly different. In addition, Kd values of RA cells treated with either 90-HA or 190-HA decreased compared with that of control RA cells, but these decreases were not statistically significant. On the other hand, Bmax values indicated that the synovial fibroblasts of RA possessed the maximal number of u-PA binding sites, which was about three times as many as those of OA. In OA cells, treatment with either 90-HA or 190-HA decreased the u-PA binding sites, but no significant change was observed between these treatments. On the other hand, in RA cells, stimulation with 90-HA showed a two-fold decrease in Bmax, and 190-HA decreases Bmax by a further quarter compared with that of untreated cells. Thus, 190-HA was a more potent suppressor of u-PAR expression than 90-HA in RA fibroblasts.

Effects of HA on the immunocytochemical localization of u-PAR

The expression and localization of u-PAR on the synovial fibroblasts of OA and RA were analyzed by an immunocytochemical study using monoclonal antibody against u-PAR before and after the stimulation of HAs to these cells. As shown in Fig. 3, the immunoreactive u-PAR, which was stained a greenish color, was observed in and around the synovial fibroblasts of both OA and RA in the absence of HAs, and the immunoreactivity was stronger in RA cells than in OA cells (Fig. 3A,D). When these fibroblasts were treated with HAs, the expression of u-PAR was inhibited in both cells, and the degree of inhibition was stronger in RA cells than in OA cells (Fig. 3B,C,E,F). Moreover, the inhibitory effect was greater with stimulation with 190-HA compared with that with 90-HA in RA cells (Fig. 3E,F). Therefore, the immunological effect of u-PAR is roughly associated with the maximal number of binding sites estimated by u-PA binding assay (Table 1).

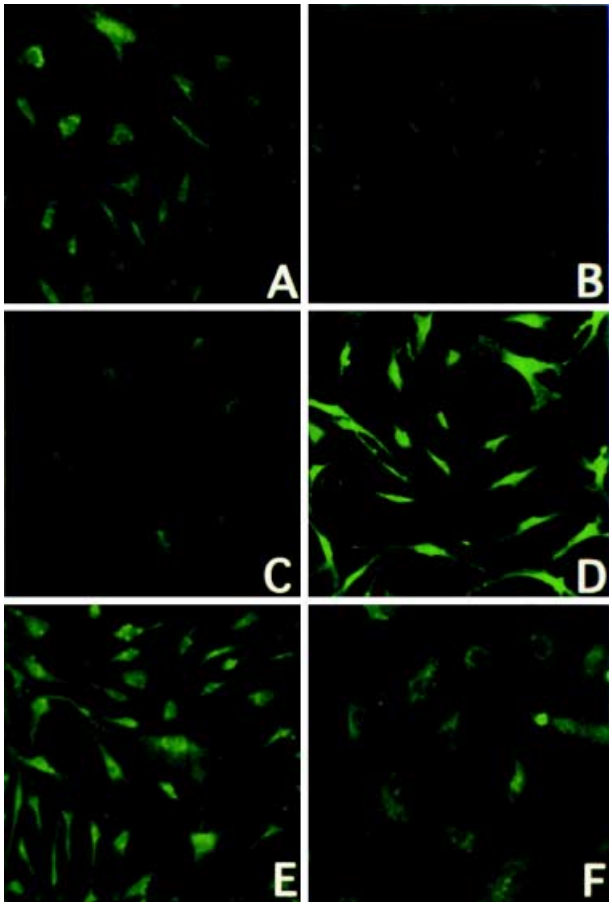

Fig. 3. Immunocytochemical studies of the effects of HA on the expression and localization of u-PAR on synovial fibroblasts of OA (**A**,**B**,**C**) and RA (**D**,**E**,**F**). The cells were incubated at 37°C for 48 h in the absence (**A**,**D**) and presence of 90-HA (**B**,**E**) and 190-HA (**C**,**F**). Original magnification, A–F ×100

Discussion

Intraarticular administration of 90-HA has been widely used for the treatment of OA, and 190-HA has now been developed for clinical use. This study has extended our knowledge of the utilities of these two types of HA.

Although we could not obtain a normal synovium to prepare the synovial fibroblasts for a normal control, we presume that they would express t-PA as well as u-PA, since t-PA is widely distributed in normal tissues. On the other hand, as shown in our study, u-PA as well as u-PAR is essential in the pathogenesis of sinovium.

Yasui et al.[36] reported that HA inhibits the secretion of prostaglandin E_2 (PGE_2) in vitro, and thus HA can inhibit the invasive activity of inflammatory cells in synovial tissues. Furthermore, the concentration of PGE_2 in human synovial fluids was decreased by intraarticular administration of HA.[37] PGE_2 is involved in the regulation of the fibrinolytic system, since it inhibits the effects of interleukin-1 (IL-1) which increases PA activity, u-PA mRNA, and PAI-2, but decreases PAI-1 in human synovial fibroblasts.[38] These phenomena may be induced by intracel-

lular signal transduction mediated by the PGE_2 pathway.[39] Therefore, our findings suggest that the changes in the levels of u-PA and PAI-1 in the presence of HAs may be associated with the PGE_2 pathway, which needs further study. The effects of both HAs (90-HA and 190-HA) on the suppression of both u-PA and PAI-1 seemed more prominent in synovial fibroblasts of OA, and the effects on PAI-1 suppression seemed more prominent in synovial fibroblasts of RA. However, the expected decrease in u-PAR expression was found in RA cells rather than in OA cells.

Szekanecz et al.[22] reported the expression of u-PAR in synovial tissues where u-PAR was expressed in 70%–90% of synovial tissue lining cells and subsynovial and interstitial macrophages in arthritis tissue, but u-PAR was present in only a few myeloid cells in normal synovial tissue. Cerinic et al.[40] showed that the number of u-PAR binding sites in synovial fibroblasts of RA was significantly higher than those in OA by means of a radiolabeled ligand binding assay, a cross-linking assay, and transmission electron microscopy of a gold–u-PA complex. We also demonstrated the presence of u-PAR in synovial fibroblasts by electrophoretic enzymography, a u-PA binding assay, and immunocytochemical analysis. Both HAs specifically reduce the expression of u-PAR in both OA and RA fibroblasts, as shown by consistent decreases in Bmax for u-PA (Table 1) and the u-PA activity which was recovered from the cell surface, together with u-PAR, by PI-PLC treatment (see Fig. 2), and the fluorescent intensity of each immunologically u-PAR-positve cell (see Fig. 3). Furthermore, HAs did not have any effects on the function of u-PAR, since the Kd values which indicate the affinity of u-PAR to its ligand u-PA were not significantly changed by HA treatment (Table 1). Thus, HA may act as a regulator of u-PAR expression of synovial fibroblasts, and 190-HA is more effective than 90-HA in RA fibroblasts.

The expression of u-PAR may reflect the pathological conditions in OA and RA. The amount of u-PAR was three times higher in RA than in OA, and this increase in u-PAR may cause an accelerated proteolysis around the cells by binding u-PA.

The two types of HA preferably decreased the secretion of u-PA, and the degree of suppression of u-PA was greater than that of PAI-1 in OA synovial fibroblasts, indicating that the fibrinolytic activity in the synovial fluids was downregulated in OA. Furthermore, these HAs also reduced the expression of u-PAR on OA fibroblasts, resulting in a significant decrease of u-PA activity which bound on the u-PAR, suggesting that the cellular fibrinolytic potential was also downregulated in OA. Thus, the two types of HA may be effective in reducing proteolytic states of synovium in OA.

Although 90-HA decreased the expression of u-PAR, it had no effect on the secretion of u-PA from RA cells. On the other hand, 190-HA significantly reduced the u-PA secretion and efficiently suppressed the expression of u-PAR in RA cells. Therefore, 190-HA was considered to be a more potent inhibitor of u-PA and u-PAR in RA cells. Although both HAs strongly reduced PAI-1 secretion from RA fibroblasts, the u-PA activity did not seem to be enhanced. The major part of PAI-1 may be a latent form that does not inactivate u-PA.[41] Therefore, the decrease of PAI-1 is likely to be negligible in the total u-PA activity.

In summary, it was suggested that both 90-HA and 190-HA could suppress the inflammatory states of synovial fibroblasts in OA and RA by reducing the expression of u-PA and/or u-PAR, and that 190-HA may be more useful than 90-HA in the treatment of RA.

Acknowledgments We would like to express our gratitude to Prof. Osamu Matsuo, Department of Physiology, Kinki University School of Medicine, and other senior colleagues for their advice and discussion.

References

1. Matsuo O, Rijken DC, Collen D. Thrombolysis by human tissue plasminogen activator and urokinase in rabbits with experimental pulmonary embolus. Nature 1981;291:590–1.
2. Collen D. On the regulation and control of fibrinolysis. Thromb Haemost 1980;43:77–89.
3. Fukao H, Ueshima S, Takaishi T, Okada K, Matsuo O. Enhancement of tissue-type plasminogen activator (t-PA) activity by purified t-PA receptor expressed in human endothelial cells. Biochim Biophys Acta 1997;1356:111–20.
4. Hagiya Y, Fukao H, Ueshima S, Okada K, Inufusa H, Kamiishi H, et al. Enhanced urokinase-type plasminogen activator activity by extracellular matrix protein obtained from highly metastatic human lung adenocarcinoma cell line. Clin Chim Acta 1996;253: 37–50.
5. Robinson RM, Taylor RE, Hansen B. Evidence for an extracellular plasmin-dependent proteolytic system in mineralizing matrices. Calcif Tissue Int 1984;36:31–8.
6. Danø K, Anderson PA, Grondahl-Hansen J, Kristensen P, Neilsen S, Skriver L. Plasminogen activators, tissue degradation and cancer. Adv Cancer Res 1985;44:139–244.
7. Sprengers ED, Kluft C. Plasminogen activator inhibitors. Blood 1987;69:381–7.
8. Barnathan ES, Kuo A, Kariko K, Rosenfeld L, Murray SC, Behrendt N, et al. Characterization of human endothelial cell urokinase-type plasminogen activator receptor protein and m-RNA. Blood 1990;76:1795–806.
9. Danø K, Behrendt N, Brunner N, Ellis V, Ploug M, Pyke C. The urokinase receptor. Protein structure and role in plasminogen activation and cancer invasion. Fibrinolysis 1994;8 Suppl 1:189–203.
10. Gardsvoll H, Danø K, Ploug M. Mapping part of the functional epitope for ligand binding on the receptor for urokinase-type plasminogen activator by site-directed mutagenesis. J Biol Chem 1999;274:37995–8003.
11. Ellis V. Functional analysis of the cellular receptor for urokinase in plasminogen activation. Receptor binding has no influence on the zymographic nature of pro-urokinase. J Biol Chem 1996;271: 14779–84.
12. Kikuchi H, Shimada W, Nonaka T, Ueshima S, Tanaka S. Significance of serine protease and matrix metalloproteinase systems in the destruction of human articular cartilage. Clin Exp Pharmacol Physiol 1996;23:885–9.
13. Nonaka T, Matsumoto H, Shimada W, Miyagi I, Okada K, Fukao H, et al. Effect of cyclic AMP on urokinase-type plasminogen activator receptor and fibrinolytic factors in a human osteoblast-like cell line. Biochim Biophys Acta 1995;1266:50–6.
14. Nonaka T, Matsumoto H, Shimada W, Okada K, Fukao H, Ueshima S, et al. Effect of bone resorbing factors on u-PA and its specific receptor in the osteosarcoma cell line. Clin Chim Acta 1993;223:129–42.
15. Leizer T, Clarris BJ, Ash PE, van Damme J, Saklatvala J, Hamilton JA. Interleukin-1 beta and interleukin-1 alpha stimulate the plasminogen activator activity and prostaglandin E_2 levels of human synovial cells. Arthritis Rheum 1987;30:562–6.

16. Clarris BJ, Leizer T, Fraser JR, Hamilton JA. Diverse morphological responses of normal human synovial fibroblasts to mononuclear leukocyte products: relationship to prostaglandin production and plasminogen activator activities, and comparison with the effects of purified interleukin 1. Rheumatol Int 1987;7:35–41.
17. Guerne PA, Zuraw BL, Vaughan JH, Carson DA, Lotz M. Synovium as a source of interleukin 6 in vitro. Contribution to local and systemic manifestations of arthritis. J Clin Invest 1989;83:585–92.
18. Butler DM, Piccoli DS, Hart PH, Hamilton JA. Stimulation of human synovial fibroblast DNA synthesis by recombinant human cytokines. J Rheumatol 1988;15:1463–70.
19. Saxne T, Lecander I, Geborek P. Plasminogen activators and plasminogen activator inhibitors in synovial fluid. Difference between inflammatory joint disorders and osteoarthritis. J Rheumatol 1993;20:91–6.
20. Pianon M, Punzi L, Stefani MP, Bertazzolo N, Michelotto M, Finco B, et al. Interleukin-1 beta, plasminogen activator and inhibitor of plasminogen activator in synovial fluid of rheumatoid arthritis, psoriatic arthritis and osteoarthritis. Agents Actions 1994;41:88–9.
21. Busso N, Peclat V, So A, Sappino AP. Plasminogen activation in synovial tissues: differences between normal, osteoarthritis, and rheumatoid arthritis joints. Ann Rheum Dis 1997;56:550–7.
22. Szekanecz Z, Haines GK, Koch AE. Differential expression of the urokinase receptor (CD87) in arthritic and normal synovial tissues. J Clin Pathol 1997;50:314–9.
23. Shimazu A, Jikko A, Iwamoto M, Koike T, Yan W, Okada Y, et al. Effects of hyaluronic acid on the release of proteoglycan from the cell matrix in rabbit chondrocyte cultures in the presence and absence of cytokines. Arthritis Rhuem 1993;36:247–53.
24. Swann DA, Radin EL, Nazimiec M, Weisser PA, Curran N, Lewinnek G. Role of hyaluronic acid in joint lubrication. Ann Rheum Dis 1974;33:318–26.
25. Tomota K. Effects of high molecular weight hyaluronon on the functions of pig phagocytes. Microbiol Immunol 1994;38:73–80.
26. Nonaka T, Kikuchi H, Ikeda T, Okamoto Y, Hamanishi C, Tanaka S. Hyaluronic acid inhibits the expression of u-PA, PAI-1, and u-PAR in human synovial fibroblasts of osteoarthritis and rheumatoid arthritis. J Rheumatol 2000;27:997–1004.
27. Nonaka T, Kikuchi H, Shimada W, Ikeda T, Hamanishi C, Tanaka S, et al. Effect of sodium hyaluronic acid on fibrinolytic factors in the synovial fluid (in vivo). Pathophysiology 1999;6:41–4.
28. Matsuo C, Fukao H, Matsuo O. Characterization of plasminogen activator produced by an established cell line from human ovary. J Cell Physiol 1988;134:253–60.
29. Nagata Y, Fukuda K, Matsumura H, Motoyoshi H, Yamasaki H, Tanaka S. Secretion of hyaluronic acid from synovial fibroblasts enhanced by histamine: a newly observed metabolic effect of histamine. J Lab Clin Med 1992;120:707–12.
30. Altman RD. Criteria for classification of clinical osteoarthritis. J Rheumatol 1991;18:10–2.
31. Arnett FC, Edworthy SM, Bloch DA. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
32. Matsuo O, Sakai T, Bando H, Okada K, Nakajima S, Takagi O, et al. Plasminogen activator in bronchoalveolar fluid. Haemostas is 1986;16:43–50.
33. Stoppelli MP, Tacchetti C, Cubellis MV, Corti A, Hearing VJ, Cassani G, et al. Autocrine saturation of pro-urokinase receptors on human A431 cells. Cell 1986;45:675–84.
34. Nonaka T, Okada K, Fukao H, Ueshima S, Kikuchi H, Tanaka S, et al. Regulation of scu-PA secretion and u-PA receptor expression in osteoblast-like cells. Cell Struct Funct 1993;18:355–62.
35. Yasuda Y, Itoh K, Mizuno N, Konishi H, Tanimura T. Alterations in migrating cranial neural crest cells in embryos of mice fed retinoic acid. Anal Cell Pathol 1989;2:23–40.
36. Yasui T, Akatsuka M, Ando M. The effect of hyaluronan on interleukin-1-α-induced prostaglandin E_2 production in human osteoarthritic synovial cells. Agents Actions 1992;37: 155–6.
37. Punzi L, Schiavon F, Todesco S. The influence of intra-articular hyaluronic acid on PGE_2 and cAMP in synovial fluid. Clin Exp Rheumatol 1989;7:247–50.
38. Hamilton JA, Wojta J, Gallichio M, Mcgrath K, Filonzi EL. Contrasting effects of transforming growth factor-β and IL-1 on the regulation of plasminogen activator inhibitors in human synovial fibroblasts. J Immunol 1993;151:5154–61.
39. Hamilton JA, Cheung D, Filonzi EL, Piccoli DS, Wojta J, Gallichio M, et al. Independent regulation of plasminogen activator inhibitor 2 and plasminogen activator inhibitor 1 in human synovial fibroblasts. Arthritis Rheum 1992;35:1526–34.
40. Cerinic MM, Generini S, Partsch G, Pignone A, Dini G, Konttinen YT, et al. Synoviocytes from osteoarthritis and rheumatoid arthritis produce plasminogen activators and plasminogen activator inhibitor-1 and display u-PA receptors on their surface. Life Sci 1998;63:441–53.
41. Aertgeerts K, Bondt HL, De Ranter CJ. Mechanism contributing to the conformational and functional flexibility of plasminogen activator inhibitor-1. Nature Struct Biol 1995;2:891–7.