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探討不同分子量玻尿酸對骨性關節炎軟骨及滑膜細胞的基質金屬蛋白水解酶-2 和-9 和胞漿素原活化劑、抑制劑及接受器之訊息傳遞路徑研究(第 2 年)

研究成果報告(完整版)

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Effects of Different Molecular Weight Hyaluronan Products on the Expression of Urokinase Plasminogen Activator and Inhibitor and Gelatinases during the Early Stage of Osteoarthritis

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ABSTRACT: Hyaluronan or hyaluronic acid (HA) has been used to treat osteoarthritic knees for more than 30 years. Here, we tested the hypothesis that HA with high molecular weight (MW) would have greater effects than HA with low MW on the expression of the plasminogen activator (PA)/plasmin system and gelatinases [matrix metalloproteinase (MMP)-2 and MMP-9] during early development of osteoarthritis (OA). We compared the levels of MMP-2, MMP-9, urokinase-type PA (u-PA), and PA inhibitor-1 (PAI-1) in a series of chondral, meniscal, and synovial cultures of early OA after treatment with or without three different MW HA products (Hyalgan and Artz with low MW, and Synvisc with high MW). Gelatin zymography revealed that three different HA products could decrease the secretion of MMP-2 in all tissue cultures and MMP-9 in meniscal and synovial cultures time-dependently. Enzyme-linked immunosorbent assay showed that Artz and Synvisc had significant inhibition on u-PA and PAI-1 levels after 24 h, but Hyalgan did at 96 h. Compared with Hyalgan and Artz, Synvisc provided the greatest ability to inhibit MMP-2, MMP-9, u-PA, and PAI-1 expression. Our studies clearly demonstrate that the therapeutic effects of using HA to treat early OA may be partially dependant on downregulation of the PA/plasmin system and gelatinases expression, which delay the structural progression of the disease. HA with high MW might have a greater ability than that with low MW to offer effective protection for articular cartilage. © 2007 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 26:475–484, 2008

Keywords: hyaluronic acid; molecular weight; osteoarthritis; plasminogen activator and inhibitor; gelatinase

INTRODUCTION

Osteoarthritis (OA), the most common form of chronic joint disorder worldwide, is characterized by progressive cartilage degeneration, subchondral bone changes, and chronic synovitis.[1] The changes of the OA knee involve not only the cartilage of articular surface but also other joint structures, such as menisci and synovia. Accumulated evidence over the past decade has demonstrated that both mechanical factors and biochemical pathways are involved in articular matrix degradation of OA.[2] Type IV collagenases (gelatinases) are members of the family of matrix metalloproteinases (MMPs) and can be divided into gelatinase-A (MMP-2) and gelatinase-B (MMP-9). They are secreted as latent precursor enzymes and can be activated by limited proteolysis, which results in a loss of molecular weight of about 10 kDa. In addition to membrane-type 1 MMP (MT1-MMP, MMP-14), MMP-2 and MMP-9 may increase expressions to play a significant role in OA pathophysiology.[3–5] With a transient primary culture model of chondral,

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meniscal, and synovial tissues acting as a system representative of the in vivo environment of early OA, proinflammatory cytokines, lipopolysaccharides, and agents which target the protein kinase C pathway, plasmin/serine proteinase, or protein synthesis can regulate the expressions of MMP-2 and MMP-9.[6]

Plasminogen activators (PAs), urokinase- (u-PA) and tissue-type PA (t-PA), are serine proteases that catalyze the conversion of the circulating zymogen, plasminogen, to generate a less specific serine protease, plasmin.[7] By single proteolytic cleavage, both u-PA and plasmin can catalyze active forms of MMPs to promote degradation of joint cartilage, such as gelatinases[8] and stromelysins.[9] PA inhibitor-1 (PAI-1) is the major circulating PAI and controls the rate of plasmin generation by forming irreversible inhibitory complexes with u-PA.[10] Interestingly, intraarticular injection of urinary trypsin inhibitor, which was shown to inhibit u-PA activity, resulted in clinical improvement in OA or rheumatoid arthritis (RA) patients.[11,12] Furthermore, MMP-2 and MMP-9, downstream enzymes of the PA/plasmin system, increase expressions in arthritic effusions to reflect the inflammatory condition of the joint.[13–15] The PA/plasmin activity and MMP-9 levels are the result of local production by either the inflammatory cells invading the affected tissue, especially neutrophils, or by synovial cells which have been stimulated as a result of inflammatory cell influx and bacterial endotoxins.[16–18] During the early development of osteoarthritis, upregulation of u-PA, PAI-1, and gelatinases expression are through three major mitogen-activated protein kinases and the phosphatidylinositol 3-kinase pathways.[19]

Based on the physiologic importance of hyaluronan [hyaluronic acid (HA)] in synovial joints, viscosupplementation of HA is used to restore the normal rheological environment which deteriorates severely in OA. Its therapeutic goal is to restore the viscoelasticity of synovial HA, decrease pain, improve mobility, and restore the natural protective functions of HA in the joint. Exogenous HA is known to downregulate MMP-3 and IL-1β,[20] decrease the secretion of both u-PA and PAI-1 in vitro,[21] inhibit the NO production,[22] delay degradation of cartilage by inhibiting glycosaminoglycan release from cartilage tissue,[23] and have antiinflammatory effects.[24] However, efficacy might be related to the rheological properties and different molecular weight (MW) of the HA which enhances penetration through the extracellular matrix (ECM) or promotes the binding to specific cell receptors, such as cluster determinant (CD)44.[25] Likewise, more studies are required to ascertain mechanisms of protective effects on OA cartilage of different MW HA in vivo. In particular, HA has been shown not to inhibit the ability of MMP-2 and MMP-9 to degrade gelatin.[26] Therefore, performing an ex vivo study to mimic in vivo environment, we tested the hypothesis that HA inhibits u-PA, PAI-1, MMP-2, and MMP-9 expression during the early stage of OA. We also tested the suppressive efficacy of different MW HA products on u-PA, PAI-1, MMP-2, and MMP-9 expression in early OA.

MATERIALS AND METHODS

Chemicals and Reagents

Three different MW HA products (sodium hyaluronate, Hyalgan®, MW = 500–730 kDa; sodium hyaluronate, Artz®, MW = 600–1,200 kDa; and chemically-crosslinked Hylan G-F 20, Synvisc®, MW = 6,000 kDa), available in Asia, the European Union, and the USA, were obtained from Fidia farmaceutici s.p.a. (Abano Terme, Italy), Seikagaku Corp. (Tokyo, Japan), and Genzyme Biosurgery (Ridgefield, NJ), respectively. All culture materials were purchased from Gibco (Grand Island, NY). All HA products were directly dissolved in the culture medium (Dulbecco's modified Eagle's medium, DMEM) and subsequently further diluted to achieve the final concentration. The concentration of HA in synovial fluid of normal adult human is 2–4 mg/ml.[27] Based on in vitro studies of other laboratories,[21,28,29] the final concentration of HA used in this study was 10 μg/ml. The concentration of HA did not induce cell death and therefore should not cause cytotoxicity or apoptosis in osteoarthritic chondral, meniscal, and synovial cultures.

Chondral, Meniscal, and Synovial Cultures

Specimens of over 250 mg from diseased cartilage, torn menisci, and hypertrophic synovia, that were all small fragments after arthroscopic debridement from five patients, including two men (aged 55 and 74) and three women (aged 53, 65, and 72), with primary early OA knees (fulfilled the American College of Rheumatology criteria and corresponded to grade II–III in the Kellgren and Lawrence classification system) by the same author at our hospital.[4,6,19,30] The remainder of the specimens were subjected to pathological examination to confirm the diagnosis. No patient had received intraarticular steroid or HA injections within the last 3 months before the surgical procedure. All patients gave informed consent for their surgical specimens to be studied. This study was conducted in accordance with the principles embodied in the Declaration of Helsinki and was approved by the Institutional Review Board of the Chung Shan Medical University Hospital of Taichung, Taiwan. Diseased tissue from each patient was divided into five groups (one control and four study groups), equally weighted at 50 mg, transferred into 24-well

culture dishes, respectively, and then incubated at 37°C under a humidified atmosphere of 5% CO_2 in DMEM supplemented with 2% penicillin-streptomycin (10,000 U/ml) and 10 mg/ml streptomycin. The tissue culture system employed has been used previously.[4,6,19,30]

Treatments of Different MW HA

The chondral, meniscal, and synovial tissues were cultured for 3 h and then transferred to a medium with or without three HA products, respectively. Furthermore, specimens in one study group were treated with an equal mixture of half Hyalgan (with the lowest MW) and half Synvisc (with the highest MW) ($\frac{1}{2}$ Hyalgan + $\frac{1}{2}$ Synvisc) treatment. Control cultures received DMEM without any HA. Incubations were continued for 4 days and the conditioned media collected at 3 h, 24 h, 48 h, and 96 h were subjected to gelatin zymography and enzyme-linked immunosorbent assay (ELISA) for the measurement of u-PA and PAI-1 antigens.

Gelatin Zymography

MMP-2 and MMP-9 levels were assayed by loading the conditioned medium which contained 10 μg of total protein onto a precast sodium dodecyl sulfate-polyacrylamide gel containing 0.1% gelatin followed by an electrophoresis.[31,32] After electrophoresis, gels were processed as described by Hsieh et al.[4] and Chu et al.[6] With a molecular weight marker being used as MMP calibrators, gelatin zymograms revealed that the latent form of MMP-2 (proMMP-2) migrated at 72 kDa and the latent form of MMP-9 (proMMP-9) presented at 92 kDa regions. The activated forms of MMP-2 and MMP-9 showed a loss of the propeptide of about 10 kDa, respectively. The nonstaining bands representing the activities of latent and activated forms of MMP-2 and MMP-9 were quantitatively measured by spot density measurement using a digital imaging analysis system (Alpha Innotech, Mt. Prospect, IL). Results were calculated as integrated density value (IDV), which was the sum of all the pixel values after background correction, i.e., IDV = Σ (each pixel value − background value).[4,15] The levels of MMP-2 and MMP-9 from the treated group were then expressed as optical density (% of control) in comparison with the control group.

Measurement of u-PA and PAI-1 Levels

Levels of u-PA and PAI-1 in conditioned media were measured by u-PA and PAI-1 ELISA kits from Biopool, Umea, Sweden. Of each conditioned medium, 200 μl of the sample were directly transferred to the microtest strip wells of the ELISA plate. All further procedures were performed following the manufacturer's instructions. The absorbance at 495 nm was measured in a microtest plate spectrophotometer and u-PA and PAI-1 levels were quantitated with a calibration curve using human u-PA and PAI-1 as a standard.[16,18,19]

Statistical Analysis

All assays were repeated three times to ensure reproducibility. For all of the measurements, analysis of variance (ANOVA) followed by Scheffe posteriori comparison was used to assess the differences between control and HA-treated groups except the differences of u-PA and PAI-1 levels between different time points using analysis of covariance (ANCOVA). Statistical significance was set at $p < 0.05$.

RESULTS

Effect of Individual HA on MMP-2 and 9 Levels

Figure 1 showed representative zymograms of conditioned media of osteoarthritic chondral, meniscal, and synovial cultures collected after an incubation of 3, 24, 48, and 96 h in the presence or absence of HA. The latent form of MMP-2

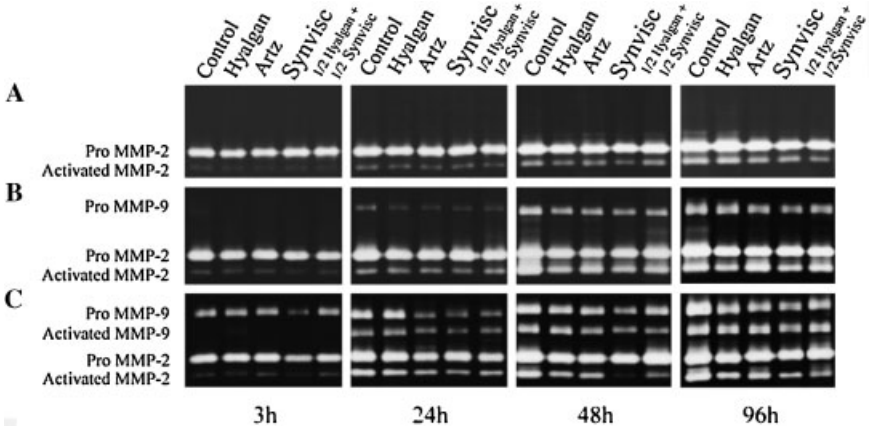

Figure 1. MMP-2 and MMP-9 levels in conditioned media from (A) chondral, (B) meniscal, and (C) synovial cultures co-treated with or without different MW HA products and HA of $\frac{1}{2}$ Hyalgan + $\frac{1}{2}$ Synvisc at 3, 24, 48, and 96 h were assayed by gelatin zymography.

(proMMP-2) appeared in all chondral, meniscal and synovial cultures, whereas the latent form of MMP-9 (proMMP-9) was observed in all synovial and some meniscal cultures; the activated MMP-9 only appeared in synovial cultures after 24 h. Although MMP-9 activation may have occurred in the meniscal cultures, its level is too low to permit detection. Bands on these zymograms were quantitated and data were shown in Table 1. The MMP-2 and MMP-9 levels were varied in the control cultures, and those from the treated cultures were expressed as percent relative to that of controls. In general, the levels of latent and activated MMP-2 in three different MW HA-treated media at 24 h seemed to have few effects in all cultures. Significantly suppressive effects of HA generally appeared at 48 h, and persisted through to 96 h, while activated MMP-2 levels in chondral ($p = 0.131$) and meniscal ($p = 0.150$) cultures of the Hyalgan-treated group, as well as proMMP-2 levels in chondral ($p = 0.096$) and meniscal ($p = 0.069$) cultures, and activated MMP-2 levels in all cultures (chondral: $p = 0.412$; meniscal: $p = 0.179$; synovial: $p = 0.059$) of the Artz-treated group did not show significant differences at 48 h. However, all of them show significant differences at 96 h. The levels of proMMP-9 in the Synvisc-treated group significantly decreased in meniscal cultures after 24 h ($p = 0.013$) and synovial cultures after 3 h ($p = 0.026$), but those in other HA-treated media did in meniscal cultures after 48 h and in synovial cultures after 24 h. The levels of activated MMP-9 in all HA-treated media significantly reduced from 48 h to 96 h. We also found that the $\frac{1}{2}$ Hyalgan $+ \frac{1}{2}$ Synvisc-treated group possessed the similar effects, and those effects seemed to be between Hyalgan- and Synvisc-treated groups. These suppressive effects were related to the duration of exposure. Thereafter, we compared the effect of different MW HA on MMP-2 and 9 levels at 96 h.

Effect of Different MW HA on MMP-2 and 9 Levels at 96 h

At 96 h, Synvisc had a significantly greater suppressive effect than Hyalgan and Artz on proMMP-2 levels in meniscal and synovial cultures, while such effect did not reach significance in chondral cultures ($p = 0.133$). Compared with Hyalgan and Artz, Synvisc seemed to have a greater suppressive effect on activated MMP-2 levels, but significant differences were only found between Synvisc-treated and Hyalgan- ($p = 0.007$) and Artz- ($p = 0.039$) treated groups in chondral cultures, and between Synvisc-treated and Hyalgan- ($p = 0.034$) treated groups in synovial cultures. The levels of proMMP-9 were more significantly inhibited by Synvisc treatment than by Hyalgan and Artz treatment in meniscal and synovial cultures, whereas activated MMP-9 levels in synovial cultures did not show significant differences between three different MW HA-treated groups ($p = 0.309$). As expected, the suppressive effects in the $\frac{1}{2}$ Hyalgan- $+ \frac{1}{2}$ Synvisc-treated group were between Hyalgan- and Synvisc-treated groups. According to these findings, we observed that Synvisc with the highest MW possess higher suppressive effects on MMP-2 and MMP-9 secretion than Hyalgan and Artz in tissue cultures of OA knees.

Effect of Different MW HA on u-PA and PAI-1 Levels

The levels of u-PA in three different MW HA-treated media at 3 h did not show significant differences in all cultures except that Synvisc had significant inhibition in synovial cultures ($p = 0.027$) (Fig. 2). Significantly suppressive effects appeared after 24 h in Artz- and Synvisc-treated media, while Hyalgan significantly decreased the u-PA levels at 96 h. Analogously, the levels of PAI-1 in all cultures showed the similar effects that Artz and Synvisc had significant inhibition after 24 h while Hyalgan did at 96 h (Fig. 3). Additionally, Synvisc showed significantly greater inhibition than Hyalgan on u-PA and PAI-1 levels in all cultures after 24 h. Synvisc also had greater inhibition than Artz on u-PA levels in meniscal cultures after 48 h and in synovial cultures at 96 h, as well as on PAI-1 levels in chondral and synovial cultures at 96 h. The $\frac{1}{2}$ Hyalgan- $+ \frac{1}{2}$ Synvisc-treated group, as expected, showed the inhibition between Hyalgan- and Synvisc-treated groups. According to these findings, we found an MW-dependent effect of HA on u-PA and PAI-1 secretion in tissue cultures of OA knees. Regarding the ratio of PAI-1 to u-PA between the control group and three different HA-treated groups, we only found significant differences in chondral cultures at 24 h ($p < 0.01$) and in meniscal cultures at 24 ($p = 0.035$) and 96 h ($p < 0.001$). In meniscal cultures at 96 h, all three HA significantly increased the ratio of PAI-1 to u-PA ($p < 0.05$) and this increase in Synvisc-treated media was significantly stronger than that in Hyalgan- ($p < 0.05$) and Artz- ($p < 0.05$) treated media. However, we could not observe any trends of modification. We also found that the inhibitory

Table 1. Levels of MMP-2 and MMP-9 in Chondral, Meniscal, and Synovial Cultures after Treatment with or without Different MW HA Products[a]

	Cartilage				Menisci				Synovia			
	Hyalgan	Artz	Synvisc	½ Hyalgan + ½ Synvisc	Hyalgan	Artz	Synvisc	½ Hyalgan + ½ Synvisc	Hyalgan	Artz	Synvisc	½ Hyalgan + ½ Synvisc
ProMMP-2 (% of control)												
3 h	100.37 ± 3.05	102.47 ± 3.95	90.53 ± 2.00	91.03 ± 6.72	101.87 ± 2.68	99.37 ± 3.51	91.10 ± 2.21	92.67 ± 1.82	103.00 ± 5.09	105.40 ± 4.55	96.73 ± 2.83	94.27 ± 2.18
24 h	93.33 ± 1.90	95.57 ± 3.10	83.77 ± 4.71^{b}	83.40 ± 5.57	94.53 ± 3.53	91.93 ± 4.82	87.13 ± 3.35^{b}	89.77 ± 2.70^{b}	96.23 ± 2.02	97.83 ± 2.15	$84.17 \pm 2.68^{b,c}$	87.10 ± 4.08^{b}
48 h	$86.10 \pm 4.96^{b,c}$	88.90 ± 0.61^{c}	$75.87 \pm 5.80^{b,c}$	76.00 ± 7.21^{b}	$87.13 \pm 4.02^{b,c}$	90.53 ± 3.71	83.50 ± 4.68^{b}	$83.30 \pm 1.68^{b,c}$	$90.30 \pm 1.51^{b,c}$	$89.47 \pm 3.10^{b,c,d}$	$76.30 \pm 4.04^{b,c}$	$76.40 \pm 3.58^{b,c,d}$
96 h	$78.60 \pm 4.89^{b,c,d}$	$82.53 \pm 7.84^{b,c,d}$	$70.57 \pm 5.20^{b,c,d}$	$71.30 \pm 6.99^{b,c}$	$85.37 \pm 1.85^{b,c,d}$	$89.10 \pm 2.39^{b,c}$	$74.23 \pm 4.50^{b,c,d}$	$75.47 \pm 5.44^{b,c,d}$	$87.80 \pm 3.26^{b,c}$	$80.70 \pm 1.57^{b,c,d,e}$	$68.47 \pm 5.62^{b,c,d}$	$75.10 \pm 4.80^{b,c,d}$
F value	21.362***	11.533**	23.56***	11.246**	21.023***	7.154**	23.218***	30.32***	14.292***	37.612***	42.360***	31.071***
Activated MMP-2 (% of control)												
3 h	100.03 ± 6.02	104.00 ± 11.51	87.70 ± 4.00	92.03 ± 2.37^{b}	103.83 ± 6.11	106.73 ± 8.43	96.90 ± 2.88	97.00 ± 2.87	100.43 ± 2.70	97.10 ± 4.06	91.20 ± 2.56	92.83 ± 3.02
24 h	97.10 ± 4.11	95.00 ± 7.93	86.80 ± 2.23	87.53 ± 3.06^{b}	95.43 ± 2.94	92.73 ± 3.47	$87.07 \pm 3.93^{b,c}$	90.03 ± 3.76	93.67 ± 2.41	89.10 ± 2.91	90.47 ± 1.90	91.40 ± 3.10^{b}
48 h	90.60 ± 2.52	87.47 ± 2.15	$73.07 \pm 7.89^{b,c}$	$81.60 \pm 1.45^{b,c}$	91.37 ± 2.75^{c}	88.33 ± 4.01^{c}	$82.10 \pm 2.88^{b,c}$	87.10 ± 1.75^{b}	$81.73 \pm 6.90^{b,c,d}$	80.97 ± 5.44	$73.47 \pm 5.22^{b,c,d}$	$81.13 \pm 2.41^{b,c,d}$
96 h	$86.70 \pm 3.57^{b,c}$	80.43 ± 8.38^{c}	$63.47 \pm 5.48^{b,c,d}$	$70.73 \pm 3.79^{b,c,d,e}$	$86.47 \pm 3.43^{b,c}$	$80.00 \pm 5.63^{b,c}$	$75.13 \pm 3.93^{b,c,d}$	$79.53 \pm 5.84^{b,c,d}$	$79.40 \pm 2.57^{b,c,d}$	$74.93 \pm 12.40^{b,c}$	$58.57 \pm 3.46^{b,c,d,e}$	$72.60 \pm 2.79^{b,c,d,e}$
F value	7.425**	5.023*	26.572***	58.041***	10.824**	12.233**	33.488***	16.634***	22.127***	8.046**	83.534***	53.641***
ProMMP-9 (% of control)												
3 h	N.D.	N.D.	N.D.	N.D.	101.40 ± 2.27	106.80 ± 5.25	96.03 ± 2.22	99.70 ± 2.35	99.53 ± 3.90	94.20 ± 4.56	88.23 ± 3.42^{b}	92.67 ± 2.59
24 h	N.D.	N.D.	N.D.	N.D.	94.50 ± 3.63^{c}	91.70 ± 4.89^{c}	86.73 ± 2.97^{b}	$91.03 \pm 1.91^{b,c}$	$86.33 \pm 3.60^{b,c}$	83.83 ± 7.39^{b}	78.77 ± 3.93^{b}	79.53 ± 7.15^{b}
48 h	N.D.	N.D.	N.D.	N.D.	$88.07 \pm 1.96^{b,c}$	$83.77 \pm 6.37^{b,c}$	$78.23 \pm 4.18^{b,c}$	$82.73 \pm 2.18^{b,c,d}$	$77.83 \pm 3.60^{b,c}$	$72.97 \pm 4.29^{b,c}$	$67.67 \pm 4.71^{b,c,d}$	$75.73 \pm 5.52^{b,c}$
96 h	N.D.	N.D.	N.D.	N.D.	$80.70 \pm 1.87^{b,c,d,e}$	$80.77 \pm 5.51^{b,c}$	$68.30 \pm 5.40^{b,c,d}$	$76.17 \pm 3.37^{b,c,d}$	$71.43 \pm 2.68^{b,c,d}$	$71.43 \pm 4.24^{b,c}$	$53.17 \pm 3.11^{b,c,d,e}$	$67.47 \pm 4.57^{b,c}$
F value					43.392***	14.564***	41.656***	65.113***	50.623***	21.420***	83.446***	23.637***
Activated MMP-9 (% of control)												
3 h	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
24 h	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	96.13 ± 2.39	93.63 ± 3.68	90.10 ± 1.08	90.03 ± 3.76^{b}
48 h	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	91.27 ± 1.46^{b}	82.23 ± 5.89^{b}	78.50 ± 7.14^{b}	84.23 ± 5.06^{b}
96 h	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	$82.27 \pm 4.62^{b,d,e}$	$78.57 \pm 4.20^{b,d}$	$72.60 \pm 9.91^{b,d}$	$79.23 \pm 1.32^{b,d}$
F value									24.089***	17.994**	11.874**	22.988***

N.D. not detectable (see Results).
ANOVA with Scheffe posteriori comparison was used.
[a]Values are mean ± SD of control values (control = 100%); $n \geq 3$.
[b]Significantly different, at $p < 0.05$, when compared to control.
[c]Significantly different, at $p < 0.05$, when compared to 3 h.
[d]Significantly different, at $p < 0.05$, when compared to 24 h.
[e]Significantly different, at $p < 0.05$, when compared to 48 h.
*$p < 0.05$.
**$p < 0.01$.
***$p < 0.001$.

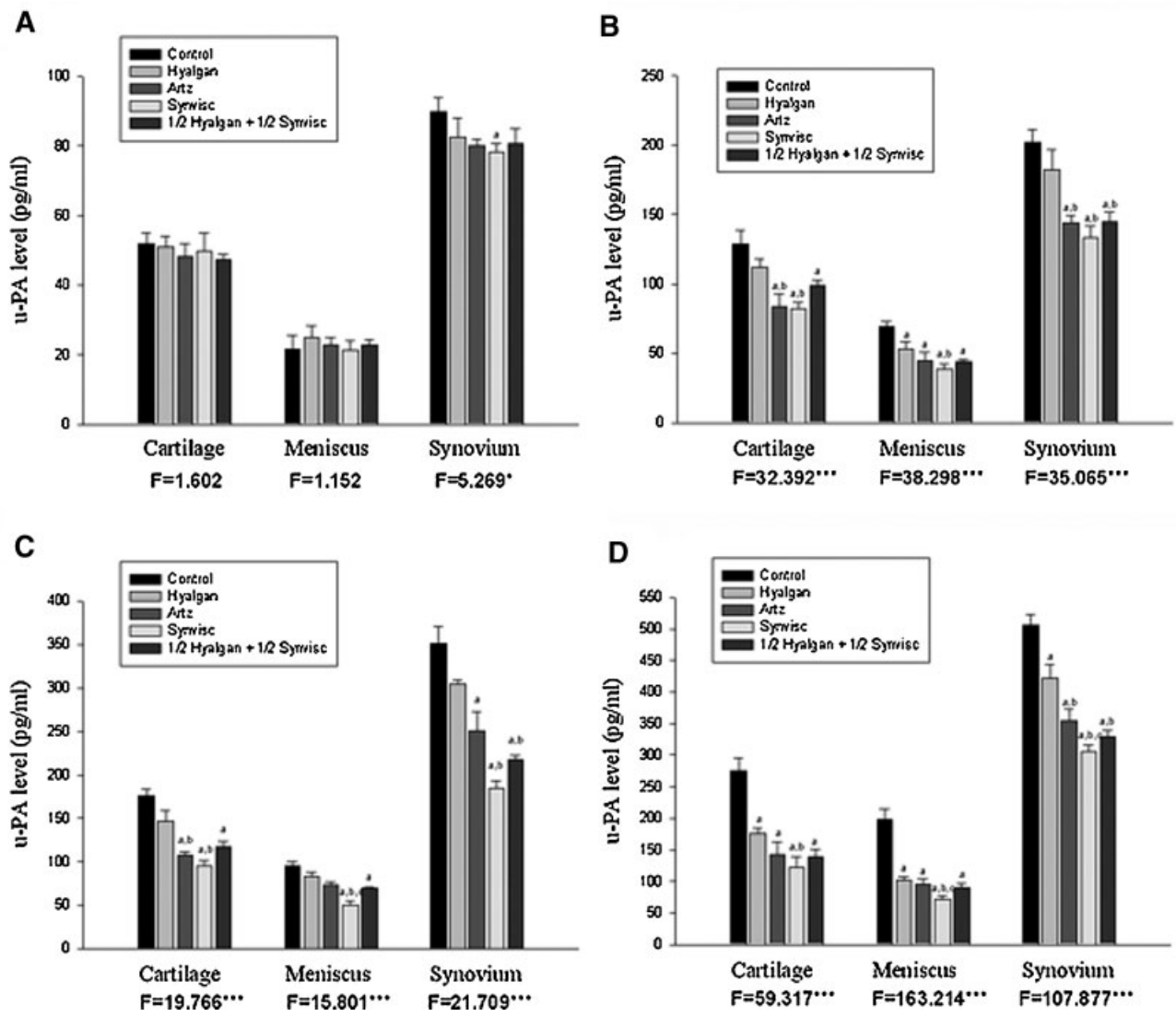

Figure 2. Levels of u-PA in chondral, meniscal, and synovial culture media after treatment with or without different MW HA and HA of $\frac{1}{2}$ Hyalgan + $\frac{1}{2}$ Synvisc at (A) 3, (B) 24, (C) 48, and (D) 96 h. Values are mean ± SD ($n \geq 3$). $^{*}p < 0.05$; $^{***}p < 0.001$. ANOVA with Scheffe posteriori comparison was used. [a]Significantly different, at $p < 0.05$, when compared to control. [b]Significantly different, at $p < 0.05$, when compared to Hyalgan. [c]Significantly different, at $p < 0.05$, when compared to Artz.

ability of three individual HA on u-PA and PAI-1 levels between different time points was not related to any trends of modification (Table 2). Synvisc had greater inhibition on u-PA and PAI-1 levels, while its inhibitory action on u-PA levels between different time points in chondral and meniscal cultures, and on PAI-1 levels in chondral cultures, did not show differences ($p > 0.05$).

DISCUSSION

The efficacy of HA products were different due to differences in physicochemical and biologic properties which could be the result of the difference in MW.[33] Given this diversity of opinion, there is, therefore, a rational basis for performing a basic study to mimic in vivo environment about the effect of different MW HA. The structure of hyaline cartilage is not uniform, but rather can be divided into distinct zones based on the arrangement of the collagen fibrils and the distribution of chondrocytes.[34] Menisci collagen fibril diameter and orientation and meniscal cell morphology vary from the surface to the deeper central regions in the meniscus. In addition to degeneration of articular cartilage, degenerative tear of the meniscus is considered to be an important and primary event of knee OA. Indeed, current evidence suggests that

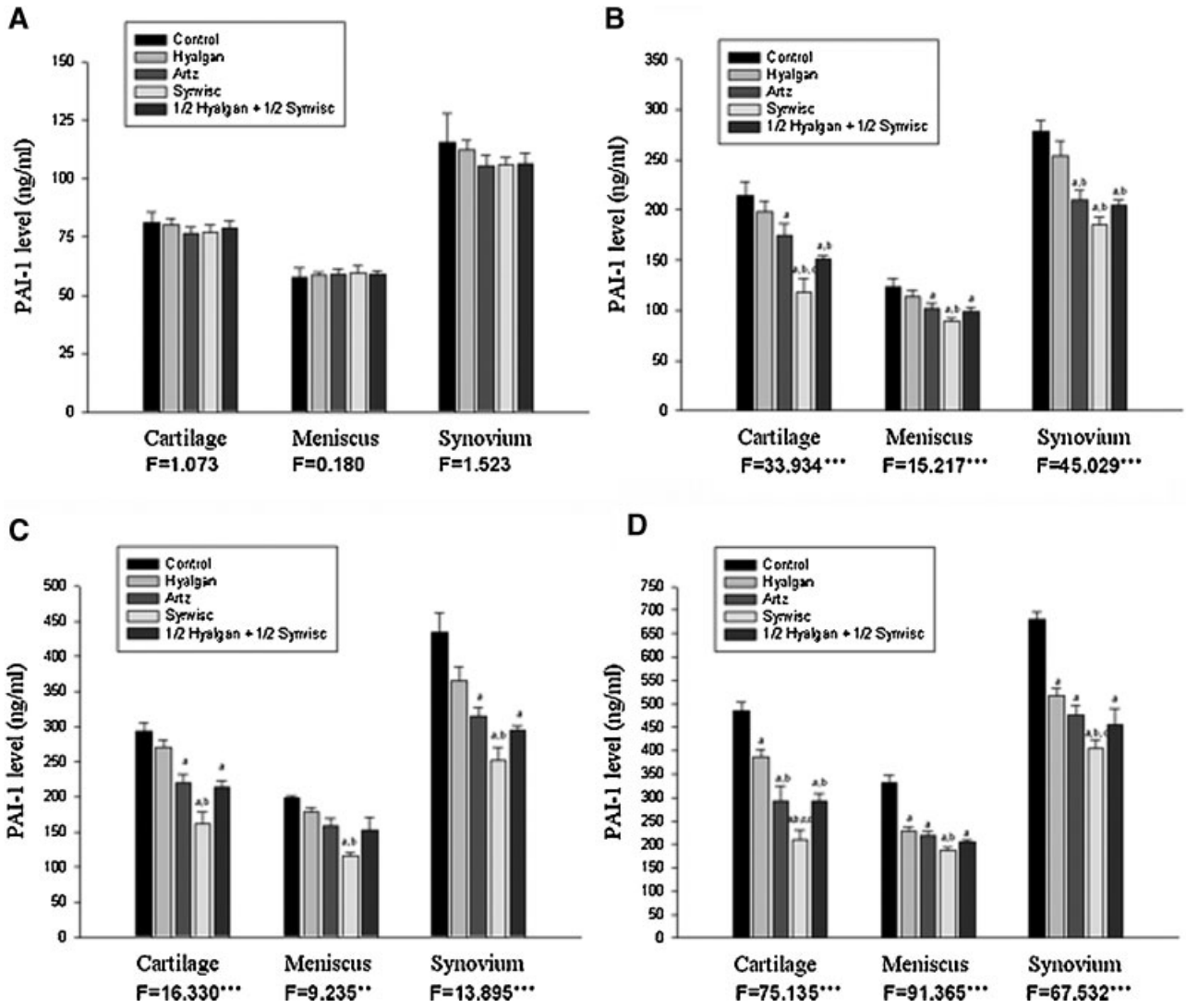

Figure 3. Levels of PAI-1 in chondral, meniscal, and synovial culture media after treatment with or without different MW HA and HA of $\frac{1}{2}$ Hyalgan + $\frac{1}{2}$ Synvisc at (A) 3, (B) 24, (C) 48, and (D) 96 h. Values are mean ± SD ($n \geq 3$). $^{**}p < 0.01$; $^{***}p < 0.001$. ANOVA with Scheffe posteriori comparison was used. [a]Significantly different, at $p < 0.05$, when compared to control. [b]Significantly different, at $p < 0.05$, when compared to Hyalgan. [c]Significantly different, at $p < 0.05$, when compared to Artz. [d]Significantly different, at $p < 0.05$, when compared to $\frac{1}{2}$ Hyalgan + $\frac{1}{2}$ Synvisc.

synovial inflammation is implicated as another central component of OA pathogenesis.[2] As discussed in our previous and the present studies,[4,6,19,30] it is important to take the meniscal and synovial tissues into account in OA.

Generally, there is a hypercoagulable and prothrombotic state with hypofibrinolysis and indirect evidence of increased fibrin generation in OA.[35] The levels of components of the PA/plasmin system in OA synovia are reported to be generally lower than those in RA synovia.[36] u-PA is indicated as the principal regulator of plasmin activity, which is able to degrade not only fibrin, but also proteins of the joint ECM and cartilage in arthritis.[37] In addition to PAIs, an increase of u-PA activity and expression of its receptor and reduced t-PA activity have been reported in joints of patients with RA and associated with the clinical severity of disease.[38] The mechanism regulating the fibrinolytic system by HA is different between OA and RA.[21] However, based on expression and modulation by antiinflammatory drugs both in OA and in animal models, u-PA in particular has been implicated in the same way as it has in RA, namely, as playing a role in inflammation and tissue remodeling.[39]

Although articular chondrocytes fail to produce MMP-9, they are not innocent bystanders in

Table 2. Inhibitory Differences of u-PA and PAI-1 Levels between Different Time Points in Chondral, Meniscal, and Synovial Cultures[a]

	Cartilage				Menisci				Synovia			
	Hyalgan	Artz	Synvisc	$\frac{1}{2}$ Hyalgan + $\frac{1}{2}$ Synvisc	Hyalgan	Artz	Synvisc	$\frac{1}{2}$ Hyalgan + $\frac{1}{2}$ Synvisc	Hyalgan	Artz	Synvisc	$\frac{1}{2}$ Hyalgan + $\frac{1}{2}$ Synvisc
u-PA level (pg/ml)												
F value	3.470	0.532	3.513	5.112	18.874**	9.160*	2.792	45.098***	0.666	0.174	11.146*	4.431
PAI-1 level (ng/ml)												
F value	3.403	3.642	1.306	6.654*	13.887**	1.724	6.128*	1.055	8.921*	0.246	6.983*	5.994*
PAI-1/u-PA												
F value	2.470	17.006**	0.981	5.974*	2.513	0.818	0.195	0.349	1.622	0.340	0.180	0.178

[a] $n \geq 3$. ANCOVA was used.
*$p < 0.05$.
**$p < 0.01$.
***$p < 0.001$.

OA.[3–6,19,30] An imbalance between the activities of MMPs and tissue inhibitor of metalloproteinase (TIMP), more increased MMPs, is thought to be important in the progression of OA, because TIMP is not elevated in OA cartilage and synovium as much as MMPs.[40] TIMP-1 expression is also found not to be influenced by HA during early development of OA, whereas HA has been shown to repress the increased MMP-3.[20] Moreover, HA does not have any direct inhibitory action on MMP-2 and MMP-9.[26] Therefore, three different MW HA products in the present study were confirmed to inhibit the levels of MMP-2 and MMP-9 secreted from the tissue cultures of early OA and their upstream enzymes of u-PA and PAI-1. Synvisc downregulates the expression of u-PA, PAI-1, MMP-2, and MMP-9 significantly more than Hyalgan and Artz. Accordingly, Synvisc provides greater inhibitory abilities to proteolysis and fibrinolysis[5,41] via inhibition of u-PA and PAI-1 levels in early OA, which are beneficial for disease modification in OA. It is likely to contribute, at least in part, to the apparent irreversibility of the OA disease process.

Two well-known characteristics of OA are a consequence of reduction in molecular size and concentration of HA in synovial fluid.[27,42] In addition to restoring the normal rheological environment, injecting exogenous HA into the knee joint enhances chondrocyte HA and proteoglycan synthesis, reduces the production and activity of proinflammatory mediators, u-PA, PAI-1, and MMPs, and alters the behavior of immune cells.[20,21,43] Both Hyalgan and Artz, extracted from rooster combs, are highly purified viscous solutions of natural HA with short intraarticular residence time (with a half-life less than 1 day).[43] The MW of Artz is slightly higher than that of Hyalgan; however, both are much lower than that of the HA in normal healthy synovial fluid.[44] Synvisc, cross-linked forms of purified HA with an extremely high MW, was developed to yield solutions with greatly enhanced elastoviscous properties like those in the knee joint of healthy young adults (18–27 years of age) and to prolong its intraarticular residence time for improving the efficacy of viscosupplementation therapy of OA. Seven days after intraarticular injection, little Synvisc remained in the synovial fluid, but significant quantities were still present in the synovial tissue and on the cartilage surface.[43] Nevertheless, the true outcomes of most of the viscosupplementation of HA are difficult to determine, because most investigators have used nebulous inclusion criteria, inadequate study designs, short-term follow-up times, and limited outcome-based analyses.[45–48]

The results of the HA therapy not only depend upon the rheological properties but also the MW of HA.[27,49] The MW-dependent binding ability to specific cell receptors, notably CD44, that allow HA to modulate cell function directly,[50] might explain the different efficacy. In normal joints, the MW of HA after production by hyalocytes has no change during the intraarticular mixing and flowing into the lymphatics of the joint capsule.[43] However, further studies are needed to know whether HA with cross-linked forms could affect its depolymerization, and degradation then affects its ability of inhibition on the expression of the PA/plasmin system and gelatinases in OA knees.

A limitation of our study was that we did not know how the efficacy of intraarticular different MW HA treatment might be influenced by the severity of OA, especially in the late stage of OA, because we targeted patients with early OA knees undergoing arthroscopic debridement and, unlike other published studies, they obtained the specimen from the late stage of OA in total knee arthroplasty. It also remains to be established whether changes observed over short-term in these ex vivo cultures would occur in vivo, because this model could increase their residence time, especially in Hyalgan- and Artz-treated cultures, and their depolymerization and degradation might be different from that in OA joints. If this chondroprotective effect could occur in vivo in joints to alter the course of OA, high MW HA products in particular may be termed disease-modifying OA drugs.[51,52]

In this study, the major findings are that (A) three different MW HA products possess the suppressive effects on MMP-2 and MMP-9 expression; (B) they also decrease u-PA and PAI-1 levels; and (C) compared with Hyalgan and Artz with lower MW, Synvisc with the highest MW provides the greatest ability to inhibit MMP-2, MMP-9, u-PA, and PAI-1 expression. Thus, learning more about the biochemical and molecular basis of ECM degradation mechanisms may help us to understand how HA affects OA knees. Intraarticular HA probably could be considered for wider use in patients with early knee OA. Certainly, they exert their effects and mechanisms on ECM proteolysis in vivo via the PA/plasmin cascade, and MMPs activation should be carefully studied.

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