High molecular weight hyaluronic acid down-regulates the gene expression of osteoarthritis-associated cytokines and enzymes in fibroblast-like synoviocytes from patients with early osteoarthritis


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Summary

Objective: Activated synoviocytes play important roles in the progression of human osteoarthritis (OA). Intra-articular injection of high molecular weight hyaluronic acid (HMW-HA) has been used as viscosupplementation for knee OA but its effect on synoviocytes remains undisclosed. This study aims to investigate the effects of HMW-HA on the gene expression of 16 OA-associated cytokines and enzymes, including interleukin (IL)-1β, IL-6, IL-8, leukemia inhibitory factor (LIF), tumor necrosis factor (TNF)-α, TNF-α converting enzyme (TACE), matrix metalloproteinase (MMP)-1, MMP-2, MMP-3, MMP-9, MMP-13, tissue inhibitor of metalloproteinase (TIMP)-1, TIMP-2, aggrecanase-1, aggrecanase-2, and inducible nitric oxide synthase (iNOS), in fibroblast-like synoviocytes (FLS) from patients with early stage OA.

Method: Synovial fluid-derived FLS were obtained from the knees of 15 patients with early stage OA. IL-1-stimulated or unstimulated FLS were cultured with or without the treatment of 600–800 kDa HMW-HA. Moreover, blocking experiments with anti-CD44 monoclonal antibodies (mAb) were used to examine the involvement of CD44 in HMW-HA effects. We designed and validated the real-time quantitative polymerase chain reaction (Q-PCR) assays with SYBR Green dyes for simultaneous quantification of the expression of the 16 genes.

Results: HMW-HA down-regulated IL-8 and iNOS gene expression in unstimulated FLS and down-regulated aggrecanase-2 and TNF-α gene expression in IL-1-stimulated FLS. CD44 binding inhibited the down-regulatory effects of HMW-HA on gene expression.

Conclusion: HMW-HA may have a structure-modifying effect for OA by down-regulation of aggrecanase-2 in FLS. HMW-HA also has an anti-inflammatory effect by down-regulation of TNF-α, IL-8, and iNOS in FLS. These effects may be mediated through the interaction of CD44 and HMW-HA.

Key words: Hyaluronic acid, Osteoarthritis, Fibroblast-like synoviocytes, Aggrecanase, Cytokine, Enzyme.
of MMP-3 and IL-1\(\beta\) in the synovium of rabbit OA model\(^{20}\). Qiu et al. found that intra-articular injection of HMW-HA decreased the mRNA expression of MMP-3 in the synovium of rabbit OA model\(^{18}\). Therefore, more studies are needed to fully elucidate the effects of HMW-HA on synoviocytes.

Reverse transcription (RT) followed by polymerase chain reaction (PCR) is the technique of choice for analyzing gene expression due to its high sensitivity. Among the various methods of quantification for RT-PCR, real-time quantitative PCR (Q-PCR) has advantages of extremely wide dynamic detection range and of higher reliability of results compared with end-point determinations using conventional PCR\(^{21}\). Real-time Q-PCR can quantify DNA fragment amplification using Taqman probes or SYBR Green fluorescence. SYBR Green is less expensive than Taqman probes and can provide an equally accurate result in real-time Q-PCR if the PCR specificity is high and the artifacts such as primer dimers are minimal\(^{22}\), thus becoming an valuable and economical tool in researching gene expression.

The aims of this study were to investigate the effects of HMW-HA on the gene expression of 16 OA-associated cytokines and enzymes, including IL-1\(\beta\), IL-6, IL-8, LIF, TNF-\(\alpha\), TACE, MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, TIMP-1, TIMP-2, aggrecanase-1, aggrecanase-2 and iNOS, in synovial fluid-derived FLS from the patients with early stage OA. Furthermore, we also examine the involvement of CD44, a major HA-binding receptor, in these effects. These may clarify the mechanism of HMW-HA in treating OA. In this study, we designed and validated the real-time Q-PCR assays with SYBR Green dye for simultaneous quantification of the expression of these 16 genes.

**Methods**

**CULTURES OF SYNOVIAL FLUID-DERIVED FLS**

The study protocol was approved by the Institutional Review Board of the National Taiwan University Hospital. Synovial fluid was aspirated from the knees of 15 patients with early stage OA (Kellgren–Lawrence grades I and II). The synovial fluid was centrifuged at 450g for 30 min, cell pellets were resuspended in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal calf serum (FCS), and incubated for 24 h at 37°C in a plastic culture flask. Non-adherent cells were washed out and medium was changed daily for the next 3 days. The remaining adherent cells were cultured for additional 2 weeks in a flask before trypsinization, and then passed to new culture flasks. Cell passages 4 through 6 were FLS, which were used for phenotypic analysis and for experiments.

**PHENOTYPIC ANALYSIS OF SYNOVIAL FLUID-DERIVED FLS**

For surface marker staining, the anti-fibroblast surface molecule monoclonal antibodies (mAb) (clone D7-FIB; Abcam, Cambridge, UK) were used. FLS (passages 4 through 6) were released from culture by trypsinization, washed once, and resuspended in DMEM containing 1% FCS. Cells were incubated with the primary antibodies D7-FIB at 4°C for 30 min. After three washes, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG mAb (Serotec, Oxford, UK) as secondary antibodies at 4°C for 30 min and then washed...
three times. Subsequently, the cells were fixed in a 1.25% paraformaldehyde solution and then analyzed using a FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The positive and negative populations were determined according to the staining of unreactive isotype-matched control IgG.

For immunocytochemistry staining, the anti-fibroblast prolyl-4-hydroxylase mAb (clone 5B5; Abcam, Cambridge, UK) were used. FLS (passages 4 through 6) in 6-well culture plates and serum starved for 24 h in DMEM containing 1% FCS to synchronize cells in a non-activating and non-proliferating phase. FLS were then cultured in DMEM containing 10% FCS and either (1) stimulated with 1 ng/ml IL-1 and treated with 100 μg/ml HMW-HA for 24 h, or (2) treated with 100 μg/ml anti-CD44 blocking mAb (clone 5F12; Lab Vision Corp., Fremont, CA, USA) for 1 h. The optimal concentration of HMW-HA (100 μg/ml) used in this study was determined according to the results of a preliminary dose-response study using 10 ng/ml, 100 μg/ml, or 1 mg/ml HMW-HA to treat the FLS from five patients (Fig. 5).

**TOTAL RNA ISOLATION, QUANTIFICATION AND RT**

FLS after treatments were lysed and total RNA was extracted with Trizol agent (Life Technologies, Rockville, MD, USA) according to the manufacturer’s protocol. Total RNA was quantified by spectrophotometry. DNase digestion was carried out using DNA-free™ (Ambion Inc., Austin, TX, USA) as per the manufacturer’s protocol. One microgram of total RNA was converted to cDNA using the SuperScript II reverse transcriptase (Invitrogen, San Diego, CA, USA) for each reaction, 4 μL 5× first-strand buffer (50 mM Tris—HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂), 2 mL of 0.1 M dTT, 5 μL RNasin, 500 μM dNTP mix, 200 pmol Oligo-dT, 25 U Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) for 1 h. This reaction was then incubated at 42° C for 1 h. The finished cDNA products were stored in aliquots at −80° C until needed.

**PRIMER DESIGN AND REAL-TIME Q-PCR ASSAYS**

The mRNA sequences were retrieved from the NCBI website. The NCBI BLAST server was used to determine primer specificity, where reported similarities above 75% to non-specific sites within the human genome were undesirable. Primers were designed to span the exon—exon boundary to eliminate the possible influence of the contamination.
of genomic DNA. Lengths of PCR products were designed to range between 100 and 220 bp. Primers were designed to have a length of 18–25 bp, 40–60% GC content, and a theoretical annealing temperature of approximately 59°C. Compatible 3’ end sequences that may cause primer dimer formation, of either the primer itself or its primer pair, were avoided by visual inspection of the candidate primer sequences. Negative water controls were also prepared for each primer set.

Platinum® SYBR® Green qPCR SuperMix (Invitrogen, Carlsbad, CA, USA) was used in all reactions. The ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was used for all real-time Q-PCRs. The PCR thermal protocol applied consisted of a 2 min 95°C denaturation step, followed by 45 repeats of a 15 s 95°C denaturation step, a 30 s 59°C annealing step and a 30 s extension step at 72°C. A melting curve analysis was performed after final amplification period via a temperature gradient from 60°C to 95°C. All real-time Q-PCR amplified products were separated on agarose gel using the appropriate DNA ladder to confirm appropriate fragment sizes and lacking of primer dimers.

STANDARD CURVES

Real-time Q-PCRs for the primer sets of 18S ribosomal RNA (rRNA), hypoxanthine phosphoribosyltransferase 1 (HPRT1), IL-1β, IL-6, IL-8, LIF, TNF-α, TACE, MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, TIMP-1, TIMP-2, aggrecanase-1, aggrecanase-2 and iNOS were tested for linearity of response by constructing standard curves. Based on the methods modified from the standard curve protocol of Whelestan et al., 1/10 dilution series of the purified PCR products was prepared in nuclease-free water and SYBR Green Q-PCR was performed for the primer sets on 10^3–10^9 copies of their corresponding cDNA PCR products as templates. The target PCR products were purified by agarose gel electrophoresis. The absolute concentration of the purified PCR products was measured by using PicoGreen dsDNA quantitation reagent (Invitrogen, Carlsbad, CA, USA). The copy numbers of the purified PCR products were calculated from the DNA concentration by taking account of the size of each specific PCR product, the mean molecular weight of the nucleotide bases (340.5 g/mol), Avogadro’s number (6.022 × 10^23), and the volume.

STATISTICAL ANALYSIS AND RELATIVE QUANTIFICATION OF GENE EXPRESSION

Relative expression software tool (REST) was used to calculate comparative gene expression levels between samples after normalization to the control housekeeping gene (18S rRNA and HPRT1) expression. Using the ABI PRISM® 7700 System software, the threshold cycle, at
which the threshold cycle value ($C_T$) was measured, was adjusted to the exponential phase of the amplification traces. The average of comparative gene expression ratio was determined from triplicate real-time Q-PCRs to take variation into account. A standard curve graph of $C_T$ vs log value of starting copy number of the sample from the dilution series was constructed. The slope of the standard curve was used to determine the amplification efficiency: 

$$\text{Efficiency} = \frac{10^{-\frac{1}{\text{slope}}}}{1}$$

If calculated amplification efficiency was more than 1, then 100% amplification efficiency was assumed. Correlation coefficients were derived from the standard linear regression. Corrected comparative gene expression ratio adjusted by amplification efficiency was calculated using REST. Pair-wise fixed reallocation randomization test provided in REST was used to distinguish statistically significant results. Significance was set at $P$-value $< 0.05$.

**Results**

**PHENOTYPIC FEATURES OF SYNOVIAL FLUID-DERIVED FLS**

FLS (passages 4 through 6) exhibited uniform spindle-shaped and fibroblast-like morphology [Fig. 1(A)]. The fibroblast phenotype of FLS (passages 4 through 6) was confirmed by analysis of surface antigen and intracellular
protein. Flow cytometric analysis showed that more than 90% of FLS expressed the fibroblast surface marker [Fig. 1 (B)]. Immunocytochemistry staining revealed the presence of fibroblast product prolyl-4-hydroxylase in the majority of FLS [Fig. 1(C)], whereas negative control staining did not display a positive reaction [Fig. 1(D)].

PRIMER DESIGN AND CONFIRMATION OF PRIMER SPECIFICITY

Designed primer sequences and expected RT-PCR product sizes are listed in Table I. The specificity of RT-PCR products was confirmed with the high resolution agarose gel electrophoresis and a single band of the desired length was shown [Fig. 2(B)]. In addition, the melting curve analysis was performed. The Q-PCR assay for each gene showed a single product with a specific melting temperature as follows: 18S rRNA, 85.3°C; HPRT1, 79.7°C; MMP-1, 80.9°C; MMP-2, 86.3°C; MMP-3, 81.3°C; MMP-9, 88.2°C; MMP-13, 80.4°C; aggrecanase-1, 89.7°C; aggrecanase-2, 84.9°C; TIMP-1, 88.1°C; TIMP-2, 86.3°C; IL-1β, 86.8°C; IL-6, 82.3°C; IL-8, 80.5°C; LIF, 83.6°C; TNF-α, 87.3°C; TACE, 78.8°C; and iNOS, 82.2°C [Fig. 2(A)]. No primer dimers were generated during the applied 45 amplification cycles of real-time Q-PCR.

Q-PCR AMPLIFICATION EFFICIENCIES AND LINEARITY

All real-time Q-PCR assays functioned optimally using Platinum® SYBR® Green qPCR SuperMix without the addition of MgCl₂ to the reagent. The standard curves of Q-PCR assays for all the 16 genes had correlation coefficients of 0.98 or higher and the amplification efficiencies were mostly beyond 90% in the investigated range from 10² to 10⁹ copies of cDNA templates (Fig. 3).

DOSE-RESPONSE STUDY

In both the FLS with and without IL-1 stimulation, adding 100 μg/ml HMW-HA obviously decreased the gene expression levels of most of the 16 OA-associated cytokines and enzymes. In comparison with 100 μg/ml HMW-HA, 1 mg/ml HMW-HA had no additional down-regulatory effect on gene expression. On the other hand, 10 μg/ml HMW-HA had no obvious down-regulatory effect on gene expression (Fig. 5). Therefore, we used 100 μg/ml HMW-HA to conduct the following experiments.

HMW-HA down-regulated IL-8 and iNOS gene expression in non-stimulated FLS

In FLS without IL-1 stimulation, adding HMW-HA decreased the expression levels of all the 16 genes. Among these, adding HMW-HA significantly decreased the IL-8 gene expression level to 3.1% (P < 0.05) and significantly decreased the iNOS gene expression level to 7.5% (P < 0.05) [Fig. 6(A)].

HMW-HA down-regulated aggrecanase-2 and TNF-α gene expression in IL-1-stimulated FLS

After IL-1 stimulation, the expression levels of 11 genes significantly increased to many folds (P < 0.05) (Fig. 4). In FLS with IL-1 stimulation, adding HMW-HA decreased the gene expression levels in most of the 16 genes. Among these, adding HMW-HA significantly decreased the aggrecanase-2 gene expression level to 33% (P < 0.05) [Fig. 6(B)]. Moreover, in the absence of HMW-HA, the TNF-α gene expression level significantly increased after IL-1 stimulation (P < 0.05) (Fig. 4), however, in the presence of HMW-HA, it did not significantly increase after IL-1 stimulation. In other words, adding HMW-HA made the increase of TNF-α gene expression induced by IL-1 stimulation become insignificant.

CD44 blocking inhibited the down-regulatory effects of HMW-HA on gene expression

In FLS with IL-1 stimulation, pretreatment with anti-CD44 blocking mAb 5F12 inhibited the down-regulation of aggrecanase-2, TNF-α, IL-8, and iNOS gene expression caused by HMW-HA. However, pretreatment with isotype-matched control IgG had no effect on the down-regulation of gene expression caused by HMW-HA (Fig. 7).
Discussion

This study showed that HMW-HA can down-regulate the gene expression of aggrecanase-2, TNF-α, IL-8, and iNOS in synovial fluid-derived FLS from the patients with early stage OA. HMW-HA down-regulated the gene expression of IL-8 and iNOS in unstimulated FLS and down-regulated the gene expression of aggrecanase-2 and TNF-α in IL-1-stimulated FLS. These findings, to our knowledge, have never been reported by other researchers.

We found that HMW-HA down-regulated the gene expression of aggrecanase-2 in IL-1-stimulated FLS. Aggrecanase-2 has been identified to be the primary aggrecanase responsible for aggrecan degradation in a murine model of OA. Thus, down-regulation of aggrecanase-2 suggests that HMW-HA may have a structure-modifying effect. We
also found that HMW-HA down-regulated the gene expression of iNOS, TNF-α, and IL-8 in FLS. In the literature, iNOS is detected in OA synovium, suggesting that increased local production of NO may contribute to the pathogenesis of inflammatory arthritis by increasing synovial blood flow and by modulating cellular function within the synovium. NO also inhibits matrix synthesis in cartilage. TNF-α is a pro-inflammatory cytokine that plays key roles in altering cartilage extracellular matrix turnover. IL-8 is a cytokine that may play a regulatory function in the inflammatory process in OA. Down-regulation of these inflammatory mediators suggests that HMW-HA may have an anti-inflammatory...
effect. In addition, we observed that HMW-HA also decreased the gene expression of MMPs and other OA-associated cytokines in FLS, although not in a significant way. Therefore, based on these results, it may be reasonable to propose the use of HMW-HA in both inflammatory and non-inflammatory status for OA patients, because in the inflammatory status, HMW-HA could down-regulate aggrecanase-2 and TNF-α, while in the non-inflammatory status, HMW-HA could down-regulate IL-8 and iNOS in FLS and thus prevent inflammation and destruction of cartilage.

In the present study, we observed that the pretreatment of FLS with anti-pan CD44 blocking mAb 5F12 could reverse the down-regulatory effects of HMW-HA on the gene expression of aggrecanase-2, TNF-α, IL-8, and iNOS. This finding suggests that the suppressive effects of HMW-HA on OA-associated cytokines and enzymes in FLS are mediated through the interaction of CD44 and HMW-HA, which is consistent with the finding of Shimizu et al. in rheumatoid synovial cells. Similar suppressive effect of HMW-HA is also found in chondrocytes. Julovi et al. found that HMW-HA effectively inhibited IL-1β-stimulated production of MMP-1, MMP-3, and MMP-13 in human articular cartilage explant, which may involve direct interaction between HA and CD44 on chondrocytes. Tanaka et al. also showed that HMW-HA suppressed MMP-1 and regulated on activation, normal T expressed and secreted (RANTES) production in chondrocytes via CD44–HA interaction. However, how HMW-HA interacts with CD44 remains undisclosed. Further studies are needed to elucidate the CD44–HA signaling pathway in OA synoviocytes.

Our results demonstrated that in FLS with IL-1 stimulation, CD44 blocking only partially inhibited the down-regulatory effects of HMW-HA on gene expression. This finding implies that in addition to interacting with CD44, there may be another mechanism for the observed inhibitory effects of HA in this system. It is possible that the HA “traps” the IL-1 in solution and so prevents IL-1 binding to its receptor.

We obtained synovial fluid-derived FLS as the source of RNA for RT-PCR in this study. Although there are many researches studying FLS in OA patients, most of the FLS were derived from the traditional synovium taken from the surgical specimens of total joint arthroplasty. This means that these FLS are derived from the synovium of the patients with advanced stage OA because only such patients require total joint arthroplasty. Therefore, these cell models only represent the FLS of advanced OA, so they may be inappropriate to study the pathophysiology of FLS in the progression of OA. It may be more logical to use the synovial tissue obtained from non-arthroplastic joint surgery of patients with early stage OA. This kind of surgery, however, is few. In a recent research, the FLS obtained from synovial fluid are shown to be phenotypically and functionally the same as those derived from the synovium of surgical specimens. This makes it possible to study the FLS from the patients with early stage arthritis, whose tissue specimens are seldom available, and also allows longitudinal studies to determine whether or not the function of FLS is altered by the disease course and therapy. We used this new cell model in studying the effects of HMW-HA on synoviocytes and thus can infer the situation in the patients with early stage OA, who are appropriate candidates to receive HMW-HA treatment, from the results of this study.

The current knowledge of the biology of FLS shows that the intriguing functional property of FLS may stem from their resemblance to bone marrow stromal cells: both cell types share common progenitors and display similar gene expression profiles. It is therefore likely that, similar to the bone marrow stromal cells, FLS may support or modulate the effector character of resident or blood-derived cells in the arthritic joint. Moreover, responses to cytokines in culture and in situ hybridization studies in diseased tissue suggest that FLS secrete MMPs more readily than other fibroblasts. Since the characteristics of FLS are somewhat different from those of other fibroblasts, it is uncertain whether the data presented in our study could be obtained with primary cultures of fibroblasts from other sources. It will be of interest to investigate the effects of HMW-HA on other fibroblasts, such as skin fibroblasts, to explore the therapeutic potential of HMW-HA in other diseases.

In summary, we applied the real-time Q-PCR assays with SYBR Green dye, which were specially designed for simultaneous quantification of the gene expression of the 16 OA-associated cytokines and enzymes with high sensitivity and specificity, in synovial fluid-derived FLS from the patients with early stage OA. Our results suggest that HMW-HA may have a structure-modifying effect for OA by down-regulation of the gene expression of aggrecanase-2 in FLS. HMW-HA also has an anti-inflammatory effect by down-regulation of the gene expression of TNF-α, IL-8, and iNOS in FLS. These effects may be mediated through the interaction of CD44 and HMW-HA. Further studies are needed to elucidate the molecular mechanism of the suppressive effects of HMW-HA on these cytokines and enzymes in OA synoviocytes.

Acknowledgments

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