OsteoArthritis and Cartilage (2006) **14,** 1237–1247

doi:10.1016/j.joca.2006.05.009

Osteoarthritis and Cartilage

International Cartilage Repair Society

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

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

C.-T. Wang M.D.†, Y.-T. Lin M.D., Ph.D.‡, B.-L. Chiang M.D., Ph.D.‡, Y.-H. Lin M.Sc.‡ and S.-M. Hou M.D., Ph.D., M.P.H.†*

† *Department of Orthopedics, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan*

‡ *Department of Pediatrics, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan*

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 blocking 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.

Introduction

Recent studies suggest that osteoarthritis (OA) is a "non-classical" inflammatory disease[1,2] and that activated synoviocytes play important roles in the progression of human OA[3,4]. Synoviocytes can produce various OA-associated cytokines and enzymes, such as 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)[5–11]. In addition, a recent study showed that fibroblast-like synoviocytes (FLS) can be obtained from synovial fluid, and the phenotype and function of synovial fluid-derived FLS are the same as those derived from the synovium of surgical specimens[12]. This cell model makes it possible to study the FLS from the patients with early stage OA, whose tissue specimens are seldom available.

Hyaluronic acid (HA), a core component of extracellular matrix, comprises a repeat of *N*-acetylglucosamine and D-glucuronic acid. HA has been identified as an important modulator in many physiological and pathological processes[13,14]. Most of these responses are mediated through HA–CD44 interaction. Moreover, HA is a critical constituent component of normal synovial fluid and an important contributor to joint homeostasis[15]. In OA, both the concentration and molecular weight of intra-articular endogenous HA are decreased[16]. In clinical practice, intra-articular injection of high molecular weight HA (HMW-HA) has been used as viscosupplementation for knee OA and its therapeutic efficacy has been verified[17]. However, the exact mechanism of HMW-HA in treating OA remains partially disclosed. Only a few studies examine the effects of HMW-HA on synoviocytes[18–20]. Sasaki *et al.* found that HMW-HA inhibited the IL-1-induced expression of MMP-1 and MMP-3 in human synovial cells[19]. Takahashi *et al.* found that intra-articular injection of HMW-HA suppressed the mRNA expression

*Address correspondence and reprint requests to: Professor Sheng-Mou Hou, M.D., Ph.D., M.P.H., Department of Orthopedics, National Taiwan University Hospital and National Taiwan University College of Medicine, No. 7, Chung-Shan South Road, Taipei 100, Taiwan. Tel: 886-2-23123456x5274; Fax: 886-2-23971266; E-mail: shengmou@ha.mc.ntu.edu.tw

Received 2 February 2006; revision accepted 10 May 2006.

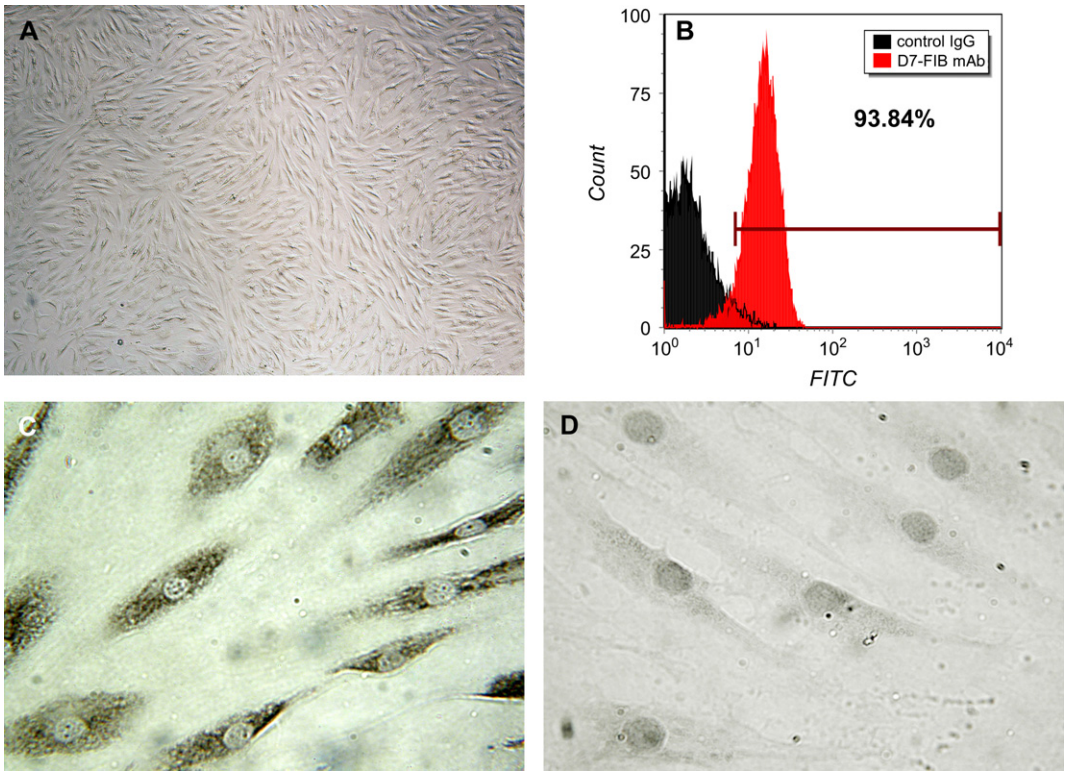

Fig. 1. Phenotypic features of synovial fluid-derived FLS (passages 4 through 6). (A) Light microscopy showed that FLS were homogeneous and spindle-shaped in morphology. (B) Flow cytometric analysis showed that more than 90% of FLS were positively stained with anti-fibroblast surface molecule mAb D7-FIB. (C) Immunocytochemistry revealed that the majority of FLS were positively stained with anti-prolyl-4-hydroxylase mAb 5B5. (D) Immunocytochemistry of negative control staining using isotype-matched control IgG did not display a positive reaction.

of MMP-3 and IL-1β 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-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, 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 450*g* 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