Hyaluronan Inhibits Tlr-4 Dependent Rankl And Cathepsin K Expression In Human Rheumatoid Arthritis Synovial Fibroblasts

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Introduction: Rheumatoid arthritis (RA) is a chronic inflammatory disease that results in synovial hyperplasia and subsequent destruction of bone and cartilage in articular joints. The receptor activator of nuclear factor-κB ligand (RANKL), a member of the tumor necrosis factor family of cytokines, is a key molecule involved in the differentiation and activation of osteoclasts. Several reports have shown that RANKL is expressed at sites of bone erosion in RA synovial membranes [1, 2]. Cathepsin K is a cysteine protease expressed by osteoclasts and synovial fibroblasts, which degrades key components of bone and cartilage such as type I and type II collagen. The positive correlation between the extent of joint destruction and the cathepsin K expression level has been reported [3]. The synovial fibroblasts play the important role in RA pathogenesis. Inhibition of RANKL and cathepsin K expression and protease activity would be important to prevent joint destruction in RA synovial membranes. Hyaluronan (HA) is a biodegradable, biocompatible, nontoxic, non-immunogenic and non-inflammatory linear polysaccharide. Some reports showed the clinical efficacy of HA intra-articular injection in RA patients. This study is designed to investigate the inhibitory effect of high molecular hyaluronan (HMW-HA) on the increased RANKL and cathepsin K expression enhanced by lipopolysaccharides (LPS) stimulation in RA synovial fibroblasts, and furthermore we studied the potential HA receptor involved in this inhibitory effect.

Methods: Patients: Five RA patients fulfilling the 1987 revised criteria of the American College of Rheumatology took part in this study. Informed consent was obtained from all patients and the experimental protocol was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine. Synovial fibroblast cells were isolated by enzymatic digestion of synovial tissue obtained from RA patients undergoing total knee arthroplasty. Cell Culture: RA synovial fibroblast cells were cultured in DMEM supplemented with 10%FBS. The cells were cultured in a 37°C humidified atmosphere. The cells were brought to overnight serum starvation, followed with 1µg/ml of lipopolysaccharide (LPS) stimulation in absence or presence of HMW-HA(ARTZ®, KAKEN, Japan) for 12 hours, and the cell lysate was then collected for analyses. Anti-TLR4 antibody (Abcam, UK) was used to block the of LPS stimulation effect. Anti-CD44 (Ancell, USA) and anti-ICAM-I (Immunotech, France) antibody were used to investigate the potential HA receptor involved in the inhibitory effect of HMW-HA on LPS-induced RANKL and cathepsin K expression. Real-time RT-PCR: Total RNA was extracted, using RNeasy Mini Kit(Qiagen, Germany). Reverse transcription was performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems,USA). Real time RT-PCR was carried out using Light cycler System with FastStart Master SYBR Green PLUS (Roche, USA) and using primers for RANKL, cathepsin K and GAPDH.
Western blotting: RANKL and cathepsin K protein expression in cell lysate was evaluated by western blotting. Total protein was extracted, using Cell Lysis Buffer (Cell Signaling, USA) containing protease inhibitor cocktail. Samples were separated by 10%SDS-PAGE gels under the reducing condition and transferred to nitrocellulose. Antibodies for RANKL (Abcam, UK), cathepsin K (Abcam, UK) and beta-actin (Cell Signaling, USA) were used for analysis.

Statistical analysis: Values were expressed as mean ± SD. Statistical comparisons were performed by the SPSS 20(IBM, USA) for Windows software. All data were analyzed by unpaired t-test. A value of p<0.05 was considered significant.

Results: Expression of RANKL enhanced by LPS via TLR-4: LPS stimulation for 12 hours increased the RANKL and cathepsin K mRNA expression in RA synovial fibroblast cells in a dose dependent manner (data not shown). Anti-TLR4 antibody clearly suppressed the RANKL and cathepsin K expression induced by LPS (Figure 1A and B).

Inhibitory effect of HMW-HA induced by LPS: Pre-incubation for 1 hour and co-incubation with 1mg/ml of HMW-HA dramatically suppressed the LPS-induced RANKL and cathepsin K mRNA expression (Figure 1A and B). This inhibitory effect showed a dose dependency. The RANKL and cathepsin K protein expression inhibitory was confirmed by western blotting analysis (Figure 2). Incubation with HMW-HA alone showed no effect on RANKL and cathepsin K expression levels (data not shown).

Inhibitory effect of HMW-HA via CD44 and ICAM-I on RANKL and cathepsin K expression induced by LPS: Pre-treatment with 5µg/ml of the anti-CD44 antibody for 1 hour significantly blocked this inhibitory effect of HMW-HA on enhanced RANKL and cathepsin K expression, while pre-treatment with anti-ICAM-I antibody did not showed significant change (Figure 3).

Discussion: In this study, we clearly demonstrated that HMW-HA suppressed the LPS-induced RANKL and cathepsin K expression. This study is the first to show the HMW-HA inhibitory effect on RANKL and cathepsin K expression in RA synovial fibroblasts. Furthermore we showed that this inhibitory effect depended on CD44-HA interaction and not on ICAM-I-HA. Previous studies reported that HMW-HA showed its anti-catabolic effect through interactions with either CD44 or ICAM-I according to the cell types and target genes [4, 5]. Although HMW-HA is used in the treatment of RA, we can find only a few reports showing that HA can prevent joints from enzymatic degradation and alter the rate of the progression of joint destruction. Our results in this study would provide the new evidence for the joint protective effect of HA injection in inflammatory arthritis.

Significance: This study is the first to demonstrate that HMW-HA inhibit the RANKL and cathepsin K expression induced by LPS via CD44-HA interaction in RA synovial fibroblasts. HA treatment would reduce the enzymatic joint destruction in inflammatory arthritis.
**Figure 1.** The effect of anti-TLR4 and HMW-HA pre- and co-incubation on LPS-induced RANKL(A) and cathepsin K (B) mRNA expression.

**Fig 2.** Western blot analysis by using RANKL and cathepsin K primary antibody with or without LPS and HA stimulation.
Figure 3. The effect of anti-CD44 and anti-ICAM-1 pre-treatment on the HMW-HA inhibitory effect for LPS induced RANKL (A) and Cathepsin K (B) mRNA expression.