Suppressive Activity of Chondroitin Sulfate on Nitric Oxide (NO) Production by Synoviocytes from Knee Osteoarthritis In Vitro

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Abstract

Background: Knee osteoarthritis (OA) is well known to be the final result of progressive alterations to articular cartilage structure, composition and cellularity. Structure-modifying medications such as intra-articular injection of hyaluronic acid or nutraceuticals, including chondroitin sulfate (CS) and glucosamine hydrochloride are also well known to be an effective treatment for OA, however, the therapeutic mechanisms of nutraceuticals are not well understood. The present study was undertaken to examine the influence of CS on the production of nitric oxide (NO), which is one of the important molecules associated with OA development, by synoviocytes from an OA patient by an in vitro cell culture technique.

Methods: Human fibroblast-like synoviocytes (1 x 10⁵ cell/ml) were stimulated with 10.0 ng/ml IL-13 in the presence of various concentrations of CS. After 48 h, NO contents in culture supernatants were examined by the Griess method. We also examined the influence of CS on transcription factor, signal transducer and activator of transcription factor 6 (STAT6), activation and inducible nitric oxide synthase (iNOS) mRNA expression in synoviocytes 12 and 24 h after IL-13 stimulation, respectively.

Results: Addition of CS into cell cultures caused the suppression of NO production from synoviocytes induced by IL-13 stimulation through the inhibition of STAT6 activation and iNOS mRNA expression. The minimum concentration of CS that caused significant suppression of NO production, STAT6 activation and iNOS mRNA expression was 7.5 μg/ml.

Conclusion: These results strongly suggest that the ability of CS to suppress NO production from synoviocytes may account, at least in part, for the clinical efficacy of CS on OA.

Introduction

Osteoarthritis of the knee (OA) is well known to be the most common joint disease in the elderly people and causes pain, stiffness, swelling and loss of function in articulating joints, which develop slowly and worsen over time [1, 2]. OA also characterizes by progressive cartilage erosion, osteophyte formation, and subchondral bone remodeling [3]. The etiology of OA is not fully understood but is currently thought to be the complex interaction of mechanical stress and inflammatory mediators and the catabolic-anabolic balance of the joint, synovium, matrix and chondrocytes [4]. Although OA is not life-threatening disease, it can deteriorate the quality of life and an economic burden through the clinical symptoms, especially pain, in affected joints during or after movement [3-5]. Biochemical observations revealed that synovial fluid obtained from OA patients contained much higher levels of matrix metalloproteinases (MMP), which are responsible for the degradation and destruction of proteoglycan and collagen in articular cartilage, as compared with healthy control and that the concentration of MMP in synovial fluids are correlated with OA severity [1, 6]. In addition to MMP, the concentration of inflammatory cytokines such as IL-1β, IL-6 and TNF-α were elevated in both synovial fluid and serum from OA patients and reflected the severity of OA, especially joint space narrowing [7, 8]. Furthermore, it is reported that nitric oxide (NO), a member of reactive oxygen species, plays essential roles in the development of OA through its activities on apoptotic cell death and tissue destruction in the joint, which are associated with cartilage matrix degradation [9, 10].

Current available treatment of OA consists of surgical and non-surgical approaches. Surgical treatments are generally considered as final procedures when non-surgical treatments failed to control several types of OA symptom, including pain and the function of the involved joint. Non-surgical treatment includes the use of nonsteroidal anti-inflammatory drugs (NSAIDs), acetaminophen and intra-articular injection of either hyaluronic acid or corticosteroids [3, 11, 12]. Physical and occupational exercises are also used frequently to prevent the development and the persistence of OA [11] as non-surgical treatments. These treatments are well known to relieve OA symptoms especially pain, stiffness, and inflammation, and improve functionally, but do not resolve the pathological changes once triggered [4, 11]. On the other hand, there are much evidence that oral administration of dietary supplements, especially chondroitin sulfate (CS) and glucosamine hydrochloride into OA patients could favorably modify the clinical conditions of the disease, including pain, stiffness and joint swelling [4, 12-14]. It is also reported the suppressive effects of CS on joint space narrowing and OA progression [15]. Although these therapeutic mode of action of agents are speculated to be owing, in part, to inhibition of prostaglandin biosynthesis and degradation of glycosaminoglycan, as well as stimulation of hyaluronic acid synthesis in the joint [15], the mechanisms by which dietary supplements could

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modify the clinical conditions of OA are not well defined. Therefore, the present study was undertaken to examine the influence of CS on NO production from synoviocytes in response to IL-13 stimulation in vitro.

Materials and Methods

Reagents

CS, purchased from WAKO Pure Chemicals Co. Ltd. (Osaka, Japan) was firstly dissolved in phosphate-buffered saline at a concentration of 1.0 mg/ml, sterilized by passing through 0.2 μm filters and further diluted with Synoviocyte Growth (SG) Medium (Cell Applications, Inc., San Diego, CA, USA) at appropriate concentrations for experiments just before use. Recombinant human IL-13 was purchased from R & D Systems, Inc. (Minneapolis, MN, USA) as a preservative free pure powder. IL-13 was also dissolved in SG Medium, sterilized and stored at -40°C until used.

Cell culture

Human fibroblast-like synoviocytes (HFLS-OA) obtained from the inflamed synovial tissues of an OA patient (Cell Applications, Inc.) was suspended in SG Medium at a concentration of 1 x 10^5 cells/ml and used as a target cell. To examine the influence of IL-13 on NO production from HFLS-OA, 1 x 10^6 cells (1.0 ml) were introduced into 24-well culture plates in triplicate and stimulated with various concentrations of IL-13 in a final volume of 2.0 ml. After 24 to 72 h, culture supernatants were collected and stored at -40°C until used. In the case of examining the influence of CS on NO production from HFLS-OA, 1 x 10^5 cells (1.0 ml) were stimulated in triplicate with 10.0 ng/ml IL-13 in the presence of 1.0 to 15.0 μg/ml CS in a final volume of 2.0 ml. After 48 h, the culture supernatants were obtained and stored at -40°C until used. To prepare cells for examining the influence of CS on transcription factor, signal transducer and activator of transcription factor 6 (STAT6), activation and inducible nitric oxide synthase (iNOS) mRNA expression in HFLS-OA after IL-13 stimulation, 1 x 10^5 cells (1.0 ml) were stimulated in triplicate with 10.0 ng/ml IL-13 in the presence of 1.0 to 15.0 μg/ml CS in a total volume of 2.0 ml for 12 and 24 h, respectively. In all experiments, CS was added to cell cultures 2 h before stimulation.

Assay for NO

NO levels in culture supernatants was examined in duplicate with commercially available Griess reagent kits, which is measurable NO, and NO, (Dojin Co., Ltd., Kumamoto, Japan) according to the manufacturer's recommended procedures.

Assay for STAT6 activation

STAT6 activity in cultured cells was analyzed by examining the levels of phosphorylated STAT6 with ELISA test kits (Abcam plc., Cambridge, MA, USA) according to the manufacturer's recommended procedures.

Assay for mRNA expression

Poly A+ mRNA was separated from cultured cells with oligo(dT)-coated magnetic micro beads (Milenyi Biotec, Bergisch Gladbach, Germany). The first-strand cDNA was synthesized from 1.0 μg of Poly A+ mRNA using a Superscript cDNA synthesis kit (Invitrogen)

Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was then carried out using a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Forster City, CA, USA). The PCR mixture consisted of 2.0 μl of sample cDNA solution (100 ng/μl), 25.0 μl of SYBR-Green Mastermix (Applied Biosystems), 0.3 μl of both sense and antisense primers, and distilled water to give a final volume of 50.0 μl. The reaction was conducted as follows: 4 min at 94°C, followed by 40 cycles of 1 min at 95°C, 1 min at 60°C and 1 min at 70°C [19]. GAPDH was amplified as an internal control. mRNA levels for iNOS were calculated by using the comparative parameter threshold cycle and normalized to GAPDH. The nucleotide sequences of the primers were as follows: for iNOS, 5’- TGCAGACACGTGCCGTACTC-3’ (sense) and 5’-GTAGCCAGCATAGGAGT-3’ (antisense), and for GAPDH, 5’-TGCACCAACACTGCTTAGC-3’ (sense) and 5’-GGCATGGACTGTGGTCATGAG-3’ [16].

Result

Influence of CS on NO production from HFLS-OA after IL-13 stimulation

The first experiments were undertaken to examine whether IL-13 stimulation could induce NO production from HFLS-OA. HFLS-OA (1 x 10^5 cells/ml) was stimulated with 10.0 ng/ml IL-13 and NO levels in culture supernatants were measured 24 to 72 h after stimulation. As shown in Figure 1A, NO levels in culture supernatants were increased, peaked at 48 h and plateaued 72 h after culture. The next experiments were designed to examine the dose response profile of IL-13 stimulation on NO production from HFLS-OA. HFLS-OA (1 x 10^5 cells/ml) was stimulated with various concentrations of IL-13 in triplicate and culture supernatants were collected 48 h after culture for measurement of NO levels. As shown in Figure 1B, stimulation of HFLS-OA with IL-13 caused significant increase in NO production from cells, which was firstly observed at 0.5 ng/ml and peaked at more than 7.5 ng/ml. The third experiments were undertaken to examine the influence of CS on NO production from HFLS-OA after IL-13 stimulation. As shown in Figure 2, treatment of cells with CS significantly suppressed NO production from HFLS-OA, which was increased by IL-13 stimulation. The minimum concentration of CS that caused significant suppression of NO production was 7.5 μg/ml.

Influence of CS on STAT6 activation in HFLS-OA after IL-13 stimulation

The fourth experiments were designed to examine the influence of CS on transcription factor, STAT6, activation in HFLS-OA in response to IL-13 stimulation. HFLS-OA (1 x 10^5 cells/ml) was stimulated with 10.0 ng/ml IL-13 in the presence of CS at concentrations of 1.0 μg/ml to 15.0 μg/ml and levels of phosphorylated STAT6 was measured 12 h after stimulation. As shown in Figure 3, treatment of cells with CS at more than 7.5 μg/ml, but not 1.0 μg/ml and 5.0 μg/ml, significantly suppressed STAT6 activation, which was increased by IL-13 stimulation.

Influence of CS on iNOS mRNA expression in HFLS-OA after IL-13 stimulation

The final experiments were carried out to examine the influence of CS on iNOS mRNA expression in HFLS-OA after IL-13 stimulation. HFLS-OA (1 x 10^5 cells/ml) was stimulated with 10.0 ng/ml IL-13 in the presence of CS at concentrations of 1.0 μg/ml to 15.0 μg/ml for 24 h.
Figure 1: Influence of IL-13 on NO production from HFLS-OA in vitro. Human fibroblast-like synoviocytes from an OA patient (HFLS-OA) at 1 x 10^5 cells/ml were cultured with different concentrations of IL-13 for 24 to 72 h. Concentration of nitric oxide (NO) in culture supernatants was measured by the Griess method. The results were expressed as the mean μM/ml ± SE of triplicate cultures. A: Time course of IL-13-induced NO production; B: dose response profile of IL-13-induced NO production; Med. alone: Medium alone. The experiments were repeated twice with similar results.

Figure 2: Influence of chondroitin sulfate (CS) on NO production from HFLS-OA after IL-13 stimulation in vitro. Human fibroblast-like synoviocytes from an OA patient (HFLS-OA) at 1 x 10^5 cells/ml were cultured with 10.0 ng/ml IL-13 for 48 h in the presence of various concentrations of CS. Nitric oxide (NO) concentration in culture supernatants was measured by the Griess method and the results were expressed as the mean μM/ml ± SE of triplicate cultures. Med. alone: Medium alone. The experiments were repeated twice with similar results.
Figure 3: Influence of chondroitin sulfate (CS) on STAT6 activation after IL-13 stimulation in vitro. Human fibroblast-like synoviocytes from an OA patient (HFLA-OA) at 1 x 10^5 cells/ml were cultured with 10.0 ng/ml IL-13 for 12 h in the presence of various concentrations of CS. Signal transducer and activator of transcription factor 6 (STAT6) activation was measured by ELISA and the results were expressed as the mean optical density at 450 nm ± SE of triplicate cultures. Med. alone: Medium alone. The experiments were repeated twice with similar results.

Figure 4: Influence of chondroitin sulfate (CS) on iNOS mRNA expression after IL-13 stimulation in vitro. Human fibroblast-like synoviocytes from an OA patient (HFLA-OA) at 1 x 10^5 cells/ml were cultured with 10.0 ng/ml IL-13 for 24 h in the presence of various concentrations of CS. Inducible nitric oxide synthase (iNOS) mRNA expression was measured by real time RT-PCR and the data expressed are the mean iNOS/GAPDH ± SE of triplicate cultures. Med. alone: Medium alone. The experiments were repeated twice with similar results.
and iNOS mRNA expression was examined by real time RT-PCR. As shown Figure 4, treatment of cells with CS at 7.5 μg/ml caused significant suppression of iNOS mRNA expression in HFLS-OA, which was increased by IL-13 stimulation. The data in Figure 4 also showed that CS at more than 10.0 μg/ml completely inhibited iNOS mRNA expression in IL-13-stimulated HFLS-OA: mRNA levels in experimental cells were nearly identical (not significant) to that in non-stimulated cells.

**Discussion**

OA is well known to be the common form of arthritis conditions and is a major cause of impaired mobility and disability among elderly individuals in developing countries [1, 4]. The main characterization of OA is a progressive destructive damage of articular cartilage, which follows alteration in the biomechanical and biochemical properties of the joints [1, 2, 6]. Since the precise mechanisms of OA progression is not fully understood, treatment focuses mostly on alleviating the symptoms of the disease rather than modifying the disease process [4, 11]. On the other hand, several reports clearly show that oral administration of glucosamine hydrochloride and CS for long periods (e.g. approximately 1 year or more) into mild- to moderate-, but not severe-, OA patients attenuate the progression of OA, including joint space narrowing and joint swelling [12-14]. However, the therapeutic mode of action of these two agents is not well defined. The present study, therefore, was undertaken to examine the influence of CS on the ability of synoviocytes to produce NO, which is one of the important molecule in the development of OA [9, 10] by using an *in vitro* cell culture technique.

The present results clearly showed that treatment of synoviocytes from an OA patient with CS significantly inhibits NO production from synoviocytes induced by IL-13 stimulation. The minimum concentration of CS that caused significant suppression was 7.5 μg/ml. After oral administration of 4000 mg CS, which is recommended standard therapeutic dose of OA [17], into healthy male volunteers, plasma CS levels gradually increased, peaked at 12.73 ± 4.69 μg/ml and decreased base line levels by 24 h [18]. It is also reported that approximately 10% to 20% of orally administered CS was absorbed [19] and reached plateau levels at 10 to 15 μg/ml in plasma, when 1200 mg CS was administered orally into healthy volunteers [19, 20]. Judging from these results, the findings of the present *in vitro* study may reflect the biological function of CS *in vivo*.

NO is well accepted to be an omnipresent intracellular messenger involved in many physiological and pathological processes [21]. NO is biosynthesized endogenously from L-arginine, oxygen and NADPH by nitric oxide synthase (NOS), namely endothelial NOS (eNOS) and neuronal NOS (nNOS). These two enzymes mediate the production of relative low levels of NO, which is responsible for maintaining homeostasis, including modulation of blood flow and neural activity [21]. On the other hand, NO produced by iNOS after inflammatory stimulation is extremely large amount and causes the oxidation of proteins, lipids and ribonucleases, among others [9, 10]. There is much evidence that NO produced in articular cartilage exerts a number of destructive events such as inhibition of both proteoglycan and collagen synthesis, proteoglycan degradation of the extracellular matrix through the activation of matrix metalloproteinases [9, 10]. It is reported that NO stimulates chondrocytes to produce interleukin-1 converting enzyme, a caspase essential for maturation of both IL-1β and IL-18, and accelerate the catabolic changes in OA tissues [9, 22]. It is also reported that NO and its derivative peroxynitrite cause chondrocyte apoptosis via a mitochondria-dependent and/or a caspase-independent mechanisms [23, 24]. Pain is well known to be the most important symptom of OA and the major determinants in functional disability [1, 4, 5]. It is reported that injection of superoxide dismutase into mice prevents not only peroxynitrite formation but also hyperalgesia associated with carrageenan injection [25], indicating that free radicals, including NO and peroxynitrite play essential roles in the development of pain and nociception. Taken together, the present results may suggest that oral administration of CS into OA patients prevents NO production from synoviocytes and results in inhibition of joint space narrowing through the inhibition of cartilage destruction and relieve pain in affected joints.

IL-13, mainly secreted from CD4+ T-helper type 2 (Th2) cells, is well known to be a multifunctional inflammatory cytokine [26] and has been shown to be a useful biomarker to monitor the efficacy of treatment and the progression of OA [2, 6, 26]. IL-13 exerts its functions through a complex receptor system, including type I and type II receptor system [27]. IL-13 binds to IL-13 receptor alpha (Ra)1 subunit, which then activates two different types of tyrosine kinases, tyrosine kinase 2 and janus kinase 2 [27, 28]. Activation of these two kinases causes dimerization of STAT6 which then translocate to the nucleus where it regulates gene expression for inflammatory mediators, including cytokines and NO [29]. The second part of experiments, therefore, was undertaken to examine the possible mechanisms by which CS could inhibit NO production from synoviocytes after IL-13 stimulation. The present data clearly showed the suppressive effects of CS at more than 7.5 μg/ml on STAT6 activation and iNOS mRNA expression in synoviocytes, which were increased by IL-13 stimulation.

**Conclusion**

The present results clearly demonstrated that CS at more than 7.5 μg/ml exerts the inhibitory effects on IL-13-induced NO production from human synoviocytes through the suppression of STAT6 activation and iNOS mRNA expression. These results strongly suggest that the ability of CS to suppress NO production from synoviocytes may account, at least in part, for the clinical efficacy of CS on OA.

**Competing Interests**

The authors declare that they have no competing interests.

**Author contribution**

Takayuki Okumo: Contribute to cell culture and sample collection. Assay for NO and STAT6 activation.

Suguru Furukawa: Assay for mRNA expression.

Hitome Kobayashi and Masataka Sunagawa:

Data analysis, including statistical analysis (for data presented in: Figures 1-4). Preparation of Figures 1-4.

Kazuhiro Asano: Contribute to study design. Participate in the methodological design and protocol. Contribute to the entire manuscript writing.

**References**

