Research Article

Influence of Glucosamine Hydrochloride on Periostin Production by Synoviocytes from Knee Osteoarthritis In vitro

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Abstract

Objective: The present study was designed to examine the influence of glucosamine hydrochloride (GH) on the ability of synoviocytes to produce periostin, which is the important effector molecule in the development of osteoarthritis (OA), in response to IL-13 stimulation in vitro.

Methods: Synoviocytes (1 x 10⁵ cells/ml) derived from an OA patient were stimulated with 10.0 ng/ml IL-13 in the presence of various concentrations of GH for 48 h. The levels of periostin in culture supernatants was examined by human periostin ELISA test kits. To examine the influence of GH on transcription factor, signal transducer and activator of transcription factor 6 (STAT6), activation and periostin mRNA expression, synoviocytes (1 x 10⁵ cells/ml) were also cultured in a similar manner for 12 and 24 h, respectively.

Results: Addition of GH into cell cultures caused the suppression of IL-13-induced periostin production from synoviocytes. The minimum concentration that caused significant suppression of periostin production was 1.0 µg/ml. GH at more than 1.0 µg/ml also inhibited STAT6 activation and periostin mRNA expression, which were increased by IL-13 stimulation in synoviocytes.

Conclusion: These results strongly suggest that GH favorably modify the clinical condition of OA patients through the suppression of periostin production after IL-13 stimulation.

ABBREVIATIONS

OA: Osteoarthritis; GH: Glucosamine Hydrochloride; SG: Synoviocyte Growth; STAT6: Signal Transducer and Activator of Transcription Factor 6; RT-PCR: Reverse Transcription-Polymerase Chain Reaction; NO: Nitric Oxide

INTRODUCTION

Knee osteoarthritis (OA) is the most common musculoskeletal diseases affecting millions of elderly people [1]. It frequently causes joint pain, tenderness, functional limitation and joint swelling, resulting in a social and economic burden [1]. Although the pathogenesis of OA is generally believed to be involved the complex interaction of mechanical stress, oxidative damage, and the catabolic-anabolic balance of the joint, synovium and matrix [2,3], the precise mechanisms of OA development are not fully understood. The current several treatments are available for OA ranging from the conservative to surgical extremes. Conservative measures involve physical therapy and pharmacological treatment with non-steroidal anti-inflammatory drugs, acetaminophen and intra-articular injection of hyaluronic acid [2,4]. These therapies are frequently used for the treatment of mild- and moderate-OA patients and results in reducing clinical symptoms such as pain and stiffness [2,4]. In contrast, irre-
versatile joints disability in sever-OA patients requires surgical replacement of the affected joints [4]. Several long-term clinical trials in OA have shown that oral administration of glucosamine hydrochloride (GH) and chondroitin sulfate favorably modify the clinical conditions of the disease such as pain, stiffness and joint swelling [5, 6]. It is also reported that these two agents inhibit joint space narrowing and OA progression [7]. Moreover, oral administration of GH at a single dose of 1000 mg/kg/day for 8 weeks into OA model rats significantly inhibits the development of erosive and degenerative changes in knee joints induced by anterior cruciate ligament transection [4]. Although the therapeutic mode of action of these two agents are speculated to be owing to chondro protective effect through inhibition of metalloproteinase activity, prostaglandin biosynthesis, and type II collagen degradation [4,5], the precise therapeutic mechanisms of these two agents are not well defined.

Periostin, an extracellular matrix protein, is secreted from various types of cells such as epithelial cells and fibroblasts after IL-4/IL-13 stimulation [8,9]. Periostin participates in normal physiological activities but also involves in pathophysiological processes in vascular diseases, wound repair, tumorogenesis and metastasis [10,11]. Periostin is also reported to play essential roles in the development of airway inflammatory diseases such as asthma, allergic rhinitis and atopic dermatitis [8,12]. In regard to periostin on OA, it is reported that periostin mRNA expression and periostin protein levels are increased in the matrix of denatured cartilage and subchondral bone in experimental animal models of OA [13,14]. Furthermore, synovial fluids obtained from OA patients contained much higher levels of periostin and its concentration gradually increases along with OA progression [15]. Immunohistochemical analysis of OA knee tissues revealed the presence of periostin in chondrocytes and lacuna located near the erosive area, which are positively correlated with severity of cartilage degeneration [16]. Although the function of periostin on the development of OA is not fully understood, these reports strongly suggest that periostin plays crucial roles in pathogenesis of OA. The present study, therefore, was undertaken to examine the influence of GH on periostin production from synoviocytes using an in vitro cell culture technique.

**MATERIALS AND METHODS**

**Reagents**

GH, purchased from SIGMA-Aldrich Co. Ltd. (St Louis, MO, USA) was dissolved in Synoviocyte Growth (SG) Medium (Cell Applications, Inc., San Diego, CA, USA) at a concentration of 1.0 mg/ml, sterilized by passing through 0.2 μm filters and stored at 4°C until used. Recombinant human IL-13 was purchased from R & D Systems, Inc. (Minneapolis, MN, USA) as preservative free pure powders. IL-13 was also dissolved in SG Medium, sterilized and stored at 4°C until used. The reagents used for mRNA isolation (TaqMan Gene Expression Cells-to-Ct™) and real-time reverse transcription-polymerase chain reaction (RT-PCR; TaqMan Gene Expression Assays) were purchased from Applied Biosystems (Foster City, CA, USA). The primers used for periostin mRNA expression was purchased from Applied Biosystems (ID: Hs01566734_m1) and 18S ribosomal RNA used as a house keeping gene was also purchased from Applied Biosystems (ID: Hs99999901_s1).

**Cell culture**

Human synoviocytes (HFLS-OA) obtained from the inflamed synovial tissues of an OA patient (Cell Applications, Inc., San Diego, CA, USA) 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 periostin production from HFLS-OA, 1 x 10^5 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 48 h, culture supernatants were collected and stored at -40°C until used. In a case of examining time course of IL-13-induced periostin production, HFLS-OA at 1 x 10^5 cells/ml was stimulated with 10.0 ng/ml IL-13 and cultured in similar manner. Culture supernatants were collected 24, 48 and 72 h after stimulation and stored at -40°C until used. To prepare cells for examining the influence of GH on transcription factor, signal transducer and activator of transcription factor 6 (STAT6), activation and periostin mRNA expression in HFLS-OA after IL-13 stimulation, 1 x 10^5 cells (1.0 ml) were introduced into each well of 24-well culture plates in triplicate. The cells were then stimulated with 10.0 ng/ml IL-13 in the presence of various concentrations of GH in a total volume of 2.0 ml for 12 and 24 h, respectively. In all experiments, GH was added to cell cultures 2 h before stimulation.

**Assay for periostin**

Periostin levels in culture supernatants were examined in duplicate by commercially available human periostin ELISA test kits (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) according to the manufacturer’s instructions. The minimum detectable level of this ELISA kit was 0.027 ng/ml.

**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 periostin mRNA expression**

mRNA expression for periostin in cultured cells was examined by real-time RT-PCR. Total RNA was isolated from 1 x 10^5 cells using 50 μL of a lysis solution (P/N 4383583) after incubation for 2 h at 37°C. Each sample of total RNA was subjected to RT using a 20x RT enzyme mix (P/N 4383585) and a 2x RT buffer (P/N 43833586) with a T100 thermal cycler (Bio-Rad Co., Hercules, CA, USA). After the RT reaction, the cDNA templates were amplified by PCR using TaqMan Gene Expression Assays, PCR primers and RT master mix (P/N 4369016). Predesigned and validated gene-specific TaqMan Gene Expression Assays [17-19] were duplicated for quantitative RT-PCR, according to the manufacturer’s protocols. PCR assays were conducted as follows: 10 min denaturation at 95°C, 40 cycles of 15s, denaturation at 95°C, and 1 min annealing and extension at 60°C. Samples were analyzed using an ABI Prism 7900HT Fast RT-PCR System (Applied Biosystems) [19,20]. Relative quantification (RQ) studies [21] were prepared from collected data [threshold cycle numbers (Ct)] with ABI Prism 7900HT Sequence-Detection System (SDS) software v. 2.3 (Applied Biosystems).
Statistical analysis

Statistical significance between control and experimental groups was examined by ANOVA followed by Dunette’s multiple comparison tests. Data analysis was performed by using ANOVA for Mac (SPSS Inc., Chicago, IL, USA). The level of significance was considered at a P value of less than 0.05.

RESULTS

Influence of GH on periostin production from HFLS-OA after IL-13 stimulation

The first experiments were designed to examine the dose response profile of IL-13 to induce periostin production from HFLS-OA in vitro. To do this, 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 periostin levels by ELISA. As shown in Figure 1A, IL-13 stimulation at 5.0 ng/ml could not increase the ability of cells to produce periostin: periostin levels in experimental cultures were nearly identical (not significant) to that in non-stimulated control. However, IL-13 at 10.0 ng/ml and more caused significant increase in periostin production from HFLS-OA. The next experiments were designed to examine the time course of periostin production from HFLS-OA after IL-13 stimulation. HFLS-OA (1 x 10^5 cells/ml) was stimulated with 10.0 ng/ml IL-13 and periostin levels in culture supernatants were measured 24 to 72 h after stimulation. As shown in Figure 1B, periostin levels in culture supernatants were gradually increased and peaked at 24 h after culture. The third experiments were undertaken to examine the influence of GH on periostin production from 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 GH at concentrations of 0.5 µg/ml to 2.0 µg/ml and periostin levels in culture supernatants were measured 48 h after stimulation. As shown in Figure 2, treatment of cells with GH at 0.5 µg/ml could not suppress periostin production from HFLS-OA in response to IL-13 simulation. However, addition of GH at more than 1.0 µg/ml into cell cultures caused complete inhibition of periostin production from HFLS-OA after IL-13 stimulation: periostin levels in experimental culture supernatants was nearly identical (not significant) to that in non-stimulated control.

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

The second set of experiments was designed to examine the influence of GH 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 GH at concentrations of 0.5 µg/ml to 2.0 µg/ml and levels of phosphorylated STAT6 was measured 12 h after stimulation. As shown in Figure 3, treatment of cells with GH at more than 1.0 µg/ml but not 0.5 µg/ml significantly suppressed STAT6 activation, which was increased by IL-13 stimulation.

Influence of GH on the periostin mRNA expression in HFLS-OA after IL-13 stimulation

The final set of experiments was carried out to examine the influence of GH on periostin 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 GH at concentrations of 1.0 µg/ml to 2.0 µg/ml for 24 h and periostin mRNA expression was examined by real time RT-PCR. As shown in Figure 4, treatment of cells with GH at 1.0 µg/ml caused significant suppression of periostin mRNA expression in HFLS-OA, which was increased by IL-13 stimulation. The data in Figure 4 also showed that GH at more than 1.5 µg/ml completely inhibited periostin 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 joint disorder and is a major cause of impaired mobility and disability in elderly people.
worldwide [1]. The most common symptoms observed in affected joints are pain, stiffness and swelling, which develop slowly and worsen overtime [1]. It is also accepted that OA is characterized by progressive cartilage erosion and joint space narrowing, which follow alteration in the biomechanical and biochemical properties of the joints [2, 3]. Currently available treatments of OA are divided into two categories; non-surgical and surgical approaches [2, 4]. In non-surgical treatments, considerable attentions have been paid to GH, glucosamine sulfate and chondroitin sulfate, which classified into symptomatic slow-acting drugs for OA showing chondro protective properties, as

Figure 2 Influence of glucosamine hydrochloride (GH) on periostin production from HFLS-OA after IL-13 stimulation in vitro. Human synoviocytes from an OA patient (HFLS-OA) at 1 x 10^5 cells/ml were cultured in triplicate with 10.0 ng/ml IL-13 for 48 h in the presence of various concentrations of GH. Periostin concentration in culture supernatants was measured by ELISA and the results were expressed as the mean ng/ml ± SE. The experiments were done at least twice with similar results. Med. alone: Medium alone.

Figure 3 Influence of glucosamine hydrochloride (GH) on STAT6 activation after IL-13 stimulation in vitro. Human synoviocytes from an OA patient (HFLS-OA) at 1 x 10^5 cells/ml were cultured in triplicate with 10.0 ng/ml IL-13 for 12 h in the presence of various concentrations of GH. STAT6 activation was measured by ELISA and the results were expressed as the mean optical density at 450 nm ± SE. The experiments were done at least twice with similar results. Med. alone: Medium alone.

Figure 4 Influence of glucosamine hydrochloride (GH) on periostin mRNA expression after IL-13 stimulation in vitro. Human synoviocytes from an OA patient (HFLS-OA) at 1 x 10^5 cells/ml were cultured in triplicate with 10.0 ng/ml IL-13 for 24 h in the presence of various concentrations of GH. Periostin mRNA expression was measured by real time RT-PCR and the data expressed are the mean relative quantity (RQ) ± SE. The experiments were done at least twice with similar results. Med. alone: Medium alone.

a good candidate for the pharmacological treatment of OA [4, 5]. However, the therapeutic mode of action of these agents is not well defined. The present study, therefore, was undertaken to examine the influence of GH on the ability of synoviocytes to produce periostin, which is one of the important molecules in the development of OA [15, 16] by using an in vitro cell culture technique.

The present results clearly showed that treatment of synoviocytes from an OA patient with GH significantly inhibits periostin production from synoviocytes induced by IL-13 stimulation. The minimum concentration of GH that caused significant suppression was 1.0 µg/ml. After oral administration of 1500 mg GH, which is recommended standard therapeutic dose of OA [22, 23], into healthy volunteers once a day for three days, plasma GH level reaches 2.0 mg/ml to 4.4 mg/ml [25], which are much higher than that inducing inhibitory action of GH on periostin production in vitro. Furthermore, it is also reported that synovial fluid obtained from OA patients treated with 1500 mg GH once a day for 14 consecutive days contained approximately 0.91 µg/ml GH [26], which was nearly identical level that caused the suppression of periostin production from synoviocytes. From these reports, the findings of the present in vitro study may reflect the biological function of GH in vivo.

IL-13, mainly secreted from CD4+ T cells [27], is well known to be one of the major instructive cytokines of the type 2 cytokine response and has been shown to be a useful prognostic biomarker of OA [15, 27]. IL-13 exerts the majority of its effect by signaling through STAT6. In response to IL-13 binding to its receptor, IL-13 receptor alpha (Ro)1 subunit, tyrosine kinase 2 and Janus kinase 2 are activated which in turn phosphorylate STAT6 [28, 29]. The phosphorylated STAT6 is then transported to the nucleus where it regulates gene expression for inflammatory mediators, including TNF-α, IL-6 and periostin, all of which have been implicated in the development of OA [30]. The second part of experiments,
therefore, was carried out to examine the possible suppressive mechanisms of GH on periostin production from synoviocytes after IL-13 stimulation. The present results clearly showed that the treatment of synoviocytes with GH at more than 1.0 µg/ml significantly suppress both STAT6 phosphorylation and periostin mRNA expression, which were increased by IL-13 stimulation.

Periostin is reported to increase mRNA expression for MMP-1, MMP-3, and MMP-13, which are thought to be essential catabolic enzymes for degradation of matrix in human OA cartilage [31], in chondrocytes [16]. On the other hand, periostin scarcely affected the expression of collagen mRNA, COL1 and COL2, expression which are responsible for collagen synthesis in cartilage, in chondrocytes [16], indicating that periostin may accelerate extracellular matrix destruction in OA without synthesizing new matrices. In addition to MMPs, periostin up regulates the ability of chondrocytes to produce IL-6 and IL-8, which are implicated in cartilage degradation through the enhancement of MMP production from chondrocytes and synoviocytes [16]. Moreover, periostin increases the production of nitric oxide (NO), which is one of the important final effector molecules in the development of OA, from synoviocytes through the enhancement of inducible NO synthase expression [16,32]. Together with these reports, the present results may be interpreted that oral administration of GH into OA patients suppresses periostin production from synoviocytes in response to IL-13 stimulation and results in attenuation of OA progression.

Although the present results clearly showed that GH at more than 1.0 µg/ml significantly inhibited periostin production from synoviocytes in response to IL-13 stimulation, the precise mechanisms by which GH could inhibit periostin production are not clear at present. Activation of tyrosine kinases, including tyrosine kinase 2 and janus kinase 2 and STAT6 phosphorylation require the increase in Ca²⁺ levels in cytosol [33]. GH is reported to inhibit the increase in intracellular Ca²⁺ levels in rat microglial cells after lipopolysaccharide stimulation [34]. From these reports, there is possibility that GH inhibits the activation of tyrosine kinases through the inhibition of increase in Ca²⁺ levels in synoviocytes after IL-13 stimulation, resulting in inhibition of periostin production.

CONCLUSION

The present results clearly demonstrated that GH at more than 1.0 µg/ml exerts the inhibitory effects on IL-13-induced periostin production from human synoviocytes. These results strongly suggest that the ability of GH to suppress periostin production from synoviocytes may account, at least in part, for osteoarthritis model. Life Sci. 2010; 86: 538-43.


Asano et al. (2016) 

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