

Suppressive Activity of Hyaluronic Acid on Adiponectin Production from Mouse Adipocytes *In vitro*

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Abstract

Background: Osteoarthritis (OA) is well known to be a chronic degenerative joint disease in elderly people, which are characterized by pain and progressive functional limitation. Since one cause of these symptoms is the fact that hyaluronic acid (HA), a naturally occurring joint lubricant, breaks down in patients with OA, viscosupplementation with intra-articular injection of HA is frequently used as one of important treatment options in OA, especially knee OA. However, the therapeutic mechanisms of this treatment are not well understood. The present study, therefore, was undertaken to examine the influence of HA on the production of adiponectin, which is a key element in the development of articular degenerative diseases, including OA from adipocytes using an *in vitro* cell culture technique.

Methods: Adipocytes derived from mouse 3T3-L1 fibroblasts were cultured in the presence of various concentrations of HA. After 24 h, adiponectin concentrations in culture supernatants were examined by ELISA. We also examined the effect of HA on transcription factor, Akt, activation and adiponectin mRNA expression in 24 h-cultured 3T3-L1 adipocytes by ELISA and real time RT-PCR, respectively.

Results: Treatment of adipocytes with HA inhibited adiponectin production through the suppression of both Akt phosphorylation and adiponectin mRNA expression. The minimum concentration that caused significant suppression was 3.0 mg/ml of HA.

Conclusion: These results strongly suggest that suppressive effects of adiponectin production from adipocytes may be partially responsible for the attenuating effects of HA on the development of clinical conditions of OA, especially knee OA.

Introduction

Osteoarthritis (OA) is the most common form of arthritis that affects over 300 million people globally [1]. OA can affect any joint, including hands, hip and spine, but preferentially affects the knee [1]. It is accepted that OA is a disabling articular disease characterized by pain, stiffness and swelling, which develop slowly and worsen overtime [2,3]. OA is also characterized by progressive cartilage erosion, osteophyte formation, and subchondral bone remodeling, which are thought to be responsible for loss of function in articulating joints [3]. Although the etiology of OA is not fully understood, there are evidence that a wide variety of risk factors, such as aging, obesity, knee injury, gender and metabolic syndrome are associated with OA disease development and progression [4,5]. Among these, metabolic syndromes including overweight and obesity are put forward as the most important risk factor in the development and progression of OA, especially knee OA [6,7].

Treatment of OA is divided into two categories: surgical and non-surgical treatment. Surgical treatments are generally believed as final procedures when non-surgical treatments failed to control OA symptoms, such as pain and the function of the involved joint. Non-surgical treatment includes the use of oral non-steroidal anti-inflammatory agents (NSAIDs) and intra-articular injection of corticosteroids [3,8,9]. In addition to these treatments, intra-articular injection of hyaluronic acid (HA), so called viscosupplementation, is frequently used for the treatment of knee OA to reduce pain, and improve joint function with remarkably success [10-12]. Although the mechanisms of this treatment is owing, in part, to replace missing joint lubricant [10-12], the precise therapeutic mechanisms of HA injection are not well defined. Synovial fluids obtained from obese patient with knee OA contained large amount of adipokines secreted from

infrapatellar fat pad, which is adipose tissue in the knee joints [13] and adipokines, especially adiponectin contributed to the progression of knee OA through the enhancement of pro-inflammatory cytokine, IL-6, production and matrix degradation [14]. It is also reported that adiponectin can increase expression of COX-2 and production of chemokines such as CXCL1 and CXCL5 in neutrophils and results in persistence of inflammatory responses [15], which are main mediators of the development of inflammatory responses. In the present study, therefore, the influence of HA on the production of adiponectin from adipocytes using an *in vitro* cell culture technique.

Materials and Methods

Differentiation of adipocytes

Murine 3T3-L1 preadipocytes were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% calf serum (Thermo Fisher Scientific, Inc.). To induce differentiation, two days after

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reaching confluence, the culture medium was changed to DMEM that contained 10% fetal calf serum (FCS; Thermo Fisher Scientific, Inc.), 0.5 mM isobutylmethylxanthine, 0.25 μ M dexamethasone, and 1.0 μ g/ml insulin (all Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and the cells were cultured for a further two days. The cells were then maintained DMEM supplemented with 10% FCS and 1.0 μ g/ml insulin for another two days. The differentiated cells were maintained in high glucose DMEM (Thermo Fisher Scientific, Inc.), which was changed every two days for 8 days and used as mature adipocytes.

Assay for adiponectin

To examine adiponectin secretion, mature adipocytes were suspended in high glucose DMEM at a concentration of 5×10^5 cells/ml. The cell suspensions (1.0 ml) were introduced into each well of 24-well culture plates in triplicate and cultured in the presence of various concentrations of HA (for human injection grade; Kaken Pharmaceutical Co., Ltd., Tokyo, Japan) in a final volume of 2.0 ml for 24 h [16,17]. Culture supernatants were then obtained and stored at -40°C until used. After washing the remaining cells three times with worm PBS, 2.0 ml high glucose DMEM was added into each well and disrupted with an ultrasonic homogenizer (Model 250A; Branson Ultrasonic Corp., Danbury, CT, USA) in ice cold water bath for 5 min. The homogenates were obtained and centrifuged at 10000 g for 10 min at 4°C . The supernatants were obtained and stored at -40°C until used. Adiponectin concentration was measured in duplicate by mouse adiponectin ELISA test kits (BioVender Lab. Med. Inc., Brno, Czech Republic) according to the manufacturer's recommendations. The minimum detectable level of this kit was 0.079 ng/ml.

Assay for cell viability

The influence of HA on cell viability was examined by MTT assay. Mature adipocytes suspended in high glucose DMEM at a concentration of 1×10^5 cells/ml (100 μ l) were introduced into each well of a 96-well culture plate that contained various concentrations of HA in a final volume of 200 μ l in triplicate. After 24 h [16,17], MTT solution (20 μ l) was added to each well and incubated for a further 3 h. MTT solution in each well was completely removed and 100 μ l DMSO was added for dissolving formazan. The absorbance at 570 nm was measured with a microplate reader (Bio-Rad, Co., Ltd., Hercules, Calif, USA).

Assay for phosphorylation of transcription factor

To assess the influence of HA on transcription factor activation, mature adipocytes were suspended in high glucose DMEM at a concentration of 5×10^5 cells/ml. The cells (1.0 ml) were then cultured in triplicate in 24-well culture plates with 1.0 to 7.0 mg/ml HA in a final volume of 2.0 ml for 24h [16,17]. Phosphorylation of transcription factor, Akt, in cultured cells was examined in duplicate by measuring the phosphorylated Akt level using commercially available ELISA test kits (Abcam plc., Cambridge, MA, USA) according to the manufacturer's recommended procedures.

Assay for adiponectin mRNA expression

To assess the influence of HA on adiponectin mRNA expression, mature adipocytes suspended in high glucose DMEM at concentration of 5×10^5 cells (1.0 ml) were cultured in triplicate with 1.0 to 7.0 mg/ml HA in a final volume of 2.0 ml for 24 h [16,17]. mRNA expression was measured by real time RT-PCR. Poly A⁺ mRNA was separated

from cultured cells with oligo (dT)-coated magnetic micro beads (Milteny Biotec, Bergisch Gladbach, Germany). The first-strand cDNA was synthesized from 1.0 μ g of Poly A⁺ mRNA using a Superscript cDNA synthesis kit (Invitrogen Corp., 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 μ l of sample cDNA solution (100 ng/ μ l), 25.0 μ l of SYBR-Green Mastermix (Applied Biosystems), 0.3 μ l of both sense and antisense primers, and distilled water to give a final volume of 50.0 μ l. The reaction was conducted as follows: 5 min at 94°C , followed by 40 cycles of 10 sec at 94°C , 30 sec at 60°C and 30 sec at 72°C and final elongation for 10 min at 72°C [18]. GAPDH was amplified as an internal control mRNA. mRNA levels for adiponectin were calculated by using the comparative parameter threshold cycle and normalized to GAPDH. The nucleotide sequences of the primers were as follows: for adiponectin, 5'-TGGATCTGACGACACCAAAA-3' (sense) and 5'-CGAATGGGTACATTGGGAAC-3' (antisense), and for GAPDH, 5'-ACCACAGTCCATGCCATCAC-3' (sense) and 5'-TCCACCACCCTGTTGCTGTA-3' (antisense) [16].

Statistical analysis

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

Results

Influence of HA on adiponectin secretion

The first experiments were undertaken to examine the influence of HA on adiponectin secretion from mature adipocytes. Cells (5×10^5 cells/ml) were cultured in the presence of 1.0 to 7.0 mg/ml HA for 24 h. Adiponectin levels in culture supernatants were examined by ELISA. As shown in Figure 1A, HA at lower than 2.0 mg/ml scarcely ($P > 0.05$) affected adiponectin secretion: adiponectin levels in experimental culture supernatants were similar to that in control supernatants. On the other hand, HA at more than 3.0 mg/ml caused a significant ($P < 0.05$) suppression of adiponectin secretion from mature adipocytes. To examine whether HA could inhibit adiponectin production and resulted in suppression of adiponectin appearance in culture supernatants, we examined levels of adiponectin in homogenates of mature adipocytes. As shown in Figure 1B, supernatants of experimental cell homogenates contained similar ($P > 0.05$) levels of adiponectin to those in control cell homogenates.

Influence of HA on cell viability

The second experiments were undertaken to examine the influence of HA on cell viability. Mature adipocytes at a concentration of 1×10^5 cells/ml were cultured in the presence of 1.0 to 7.0 mg/ml HA for 24 h. Cell viability was examined by MTT assay. As shown in Figure 2, there were no significant ($P > 0.05$) changes in cell viability between 1.0 mg/ml and 7.0 mg/ml HA.

Influence of HA on adiponectin mRNA expression

The third experiments were designed to examine the influence of HA on adiponectin mRNA expression in mature adipocytes. Cells

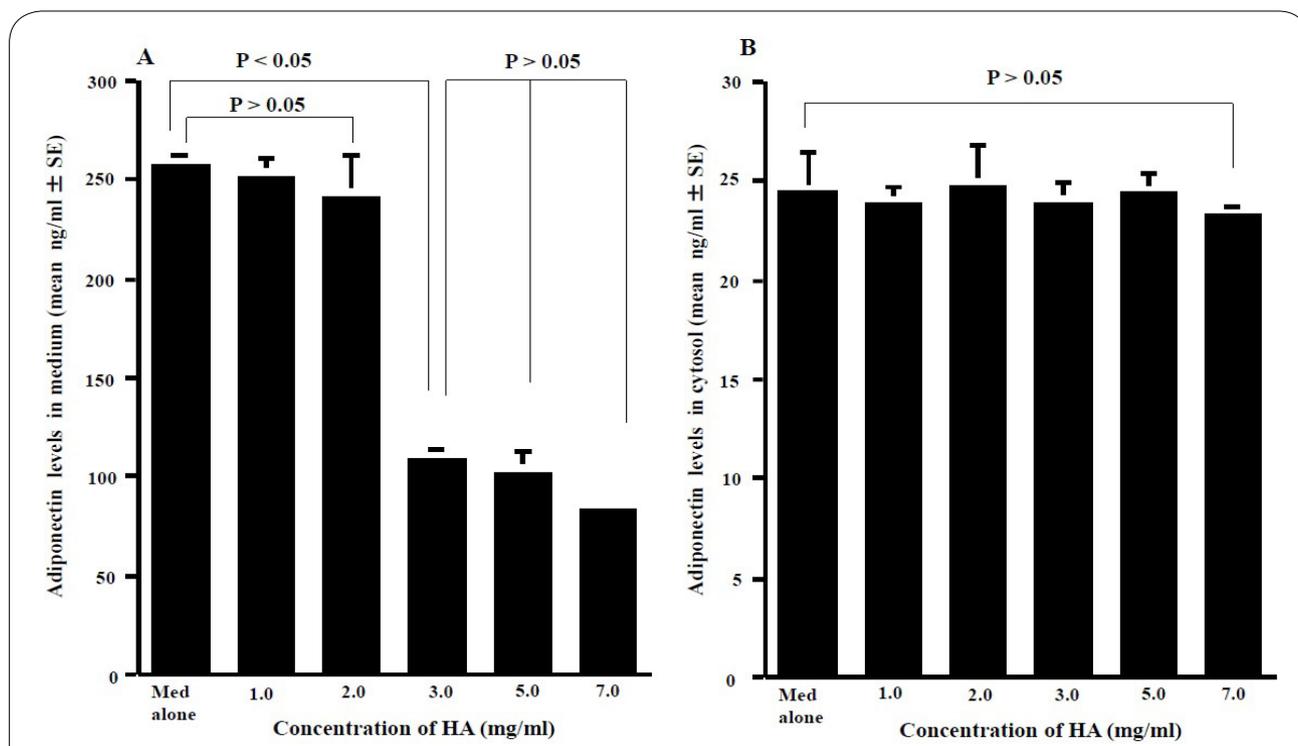


Figure 1: Influence of HA on adiponectin production from 3T3-L1 adipocytes *in vitro*. Adipocytes derived from 3T3-L1 fibroblasts at 5×10^5 cells/ml were cultured in the presence of various concentrations of hyaluronic acid (HA) for 24 h. Adiponectin levels in culture medium (A) and supernatants of cell homogenates (B) were examined by ELISA. The data are expressed as the mean ng/ml \pm SE of triplicate cultures. The experiments were done twice with similar results.

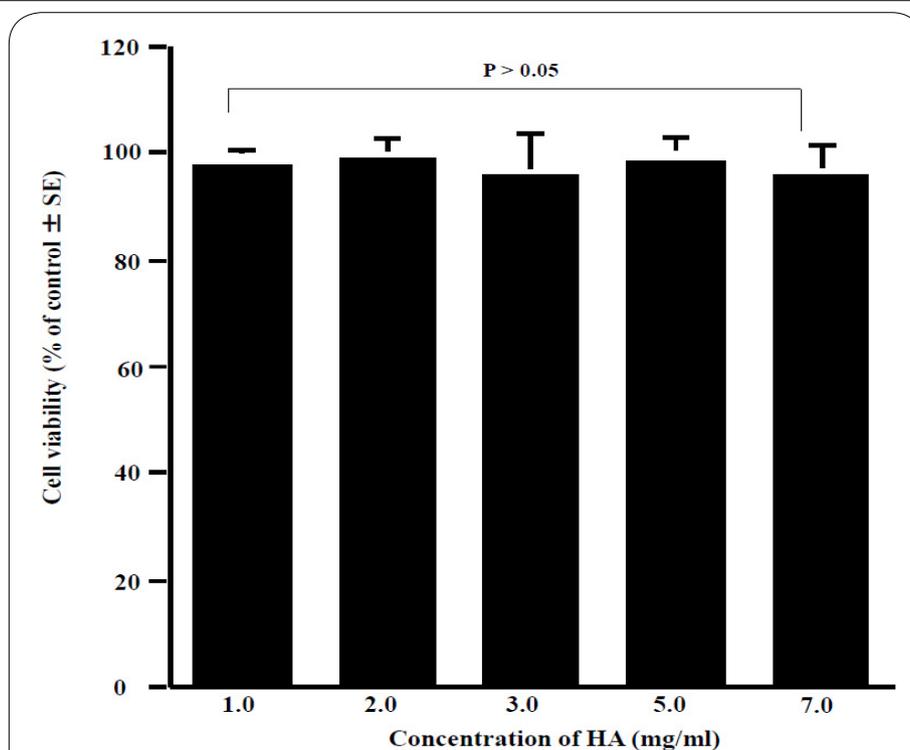
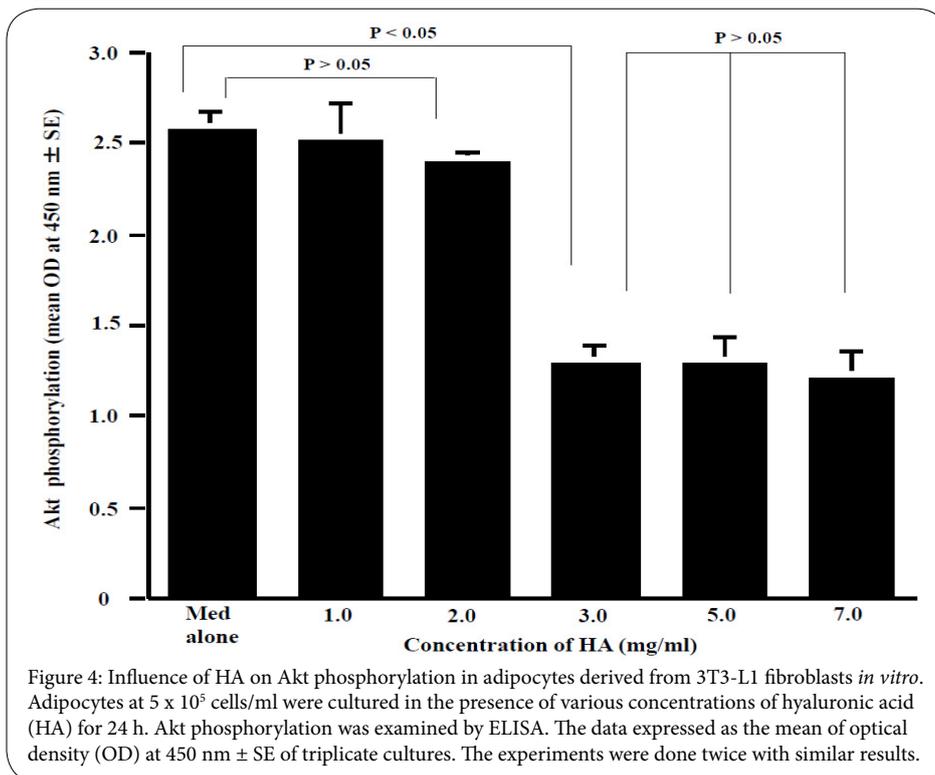
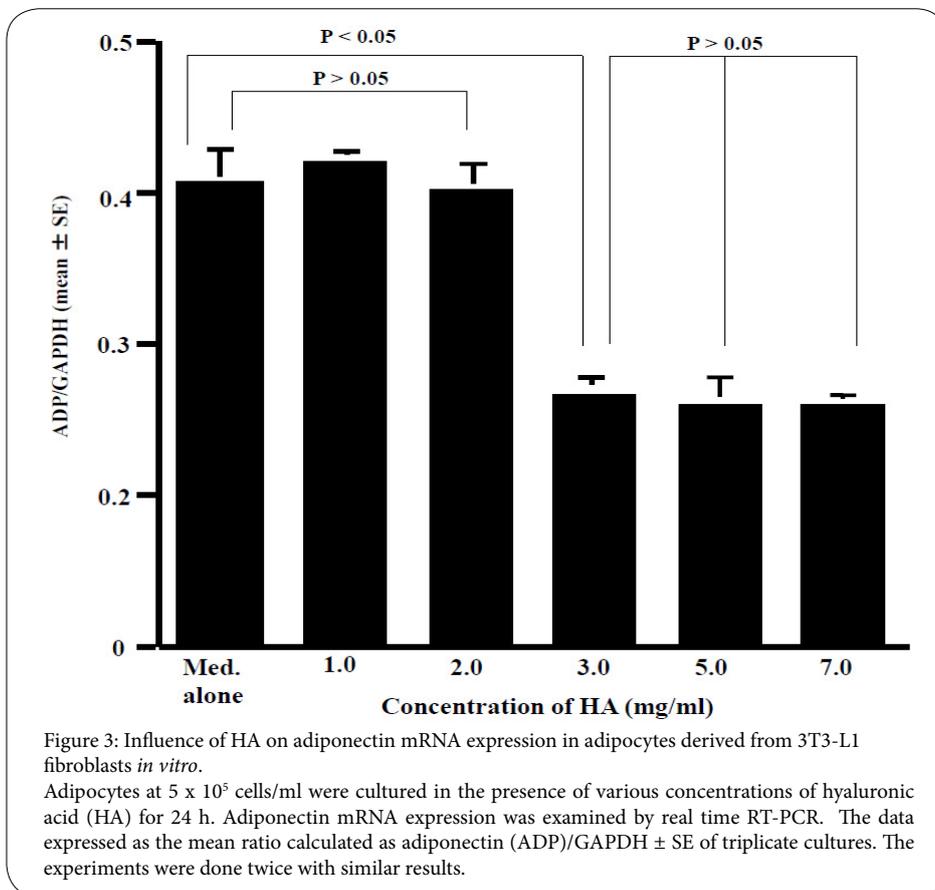


Figure 2: Influence of HA on adipocytes derived from 3T3-L1 fibroblasts *in vitro*. Adipocytes at 1×10^5 cells/ml were cultured in the presence of various concentrations of hyaluronic acid (HA) for 24 h, and then cell viability was assessed by MTT assay. The data expressed as the mean % of control \pm SE of triplicate cultures. The experiments were done twice with similar results.

(5×10^5 cells/ml) were cultured in the presence of 1.0 to 7.0 mg/ml HA for 24 h. Adiponectin mRNA expression was examined by quantitative real time RT-PCR. As shown in Figure 3, HA at more than 3.0 mg/ml,

but not less than 2.0 mg/ml, caused significant ($P < 0.05$) suppression of adiponectin mRNA expression in mature adipocytes.



Influence of HA on Akt phosphorylation

The fourth experiments were undertaken to examine the influence of HA on phosphorylation of transcription factor, Akt, in mature adipocytes. Cells (5×10^5 cells/ml) were cultured with 1.0 to 7.0 mg/ml HA for 24 h. Akt phosphorylation was examined by ELISA. As shown in Figure 4, treatment of cells with HA at more than 3.0 mg, but not less than 2.0 mg/ml significantly ($P < 0.05$) inhibited Akt phosphorylation.

Discussion

OA is well known to be a long-term chronic joint disease characterized by pain, stiffness and impaired movement in the elderly individuals [1-3]. Since the precise mechanisms of the development and progression of OA are not fully understood, current available medical treatment of OA targets mainly to relief clinical symptoms, especially pain and to delay OA progression [8,19]. It is reported that the concentration of endogenous HA in joint fluid from OA patients is lower than healthy joints [10,20], which consequently reduces the mechanical and viscoelastic activities of the synovial fluid and results in induction of pain and dysfunction of articular joints [11,12]. From these evidence, intra-articular injection of HA into the affected joint capsule is used for the treatment of OA and report the favorable modification of clinical conditions of OA, but the precise mechanisms of this therapy are not well defined [10-12]. It is reported that obesity is one of the most important contributing factors to the development and progression of OA, especially knee OA, which mainly mediated by adipokines secreted by infrapatellar fat pad [13,14]. The present study, therefore, was undertaken to examine the influence of HA on the production of adiponectin, an important member of adipokine, through the choice of adipocytes and an *in vitro* cell culture technique. The present study clearly showed that HA at more than 3.0 mg/ml significantly inhibited production, but not secretion, of adiponectin *in vitro*. It is reported that synovial fluid from healthy donors contains approximately 4 mg/ml of HA and the levels of HA in the fluid decreases 1 to 2 mg/ml in osteoarthritis, which is returned to normal levels after intra-articular HA injection [10,21]. In the treatment of knee OA, 25 mg HA injected directly into joint in a volume of 2.0 ml [22], and the half-life of HA injected is 8.8.days [10]. It is also reported that intra-articular HA injection is recommended to perform 4 times once a week [23], indicating that the concentration of HA at more than 3.0 mg in synovial fluids is maintained for about one week. Judging from these reports, suppressive effect of HA on adiponectin production observed in *in vitro* experiments may reflect the biological function of HA *in vivo*.

Adiponectin, the most important fat-derived hormone, plays critical roles in lipid and glucose metabolisms, which contributes to a beneficial metabolic effect in energy homeostasis [15,24-26]. Adiponectin exerts protective effects on a number of pathological events in various tissues and organs, including blood vessels, kidney and heart, by the inhibition of the development of inflammation, oxidative stress responses and apoptotic cell death [15,26]. On the other hand, adiponectin secreted articular adipose tissues and synovial fibroblasts, which are local producers of adiponectin [14], exerts significant destructive effects in articular tissues by inducing secretion of nitric oxide (NO), matrix metalloproteinases-3 and -9 from chondrocytes [15,25]. In addition to these extracellular matrix degradation factors, adiponectin secreted in articular cartilage is able to increase the ability of chondrocytes to produce inflammatory cytokines such as IL-6 and monocyte chemoattractant

protein (MCP)-1, which are responsible for induction of inflammatory responses [15,25]. Together with these reports, the present results strongly suggest that intra-articular HA injection favorably modify the clinical conditions of knee OA through the suppression of adiponectin levels in articular capsule.

It is reported that adiponectin production is regulated by several types of transcription factors, such as adenosine monophosphate-activated protein kinase (AMPK) and protein kinase C (PKC), among others [15,16]. Signaling pathways through Akt is reported to be a key upstream mediator for AMPK and PKC signaling to induce adiponectin production [17,27,28]. From these reports, the present results strongly suggest that treatment of adipocytes with HA causes inhibition of Akt signaling pathways and results in suppression of adiponectin production. This speculation may be supported by the observation that HA suppresses Akt phosphorylation in adipocytes at more than 3.0 mg/ml.

Although the present results clearly show that HA inhibits adiponectin production through the suppression of Akt phosphorylation, the precise suppressive mechanisms of HA on adiponectin production are not well understood. Akt phosphorylation is reported to require increase in Ca^{2+} levels in cytosol [29]. It is also reported that HA prevent increase in Ca^{2+} levels in cytosol by blocking transient receptor potential (TRP) V1 channels [30]. From these reports, there is possibility that HA inhibits Ca^{2+} influx into adipocytes and results in reducing the ability of cells to produce adiponectin. Further experimentations are required to clarify this point.

Conclusion

The present results strongly suggest that intra-articular injection of HA causes decrease in adiponectin levels in synovial fluids and results in favorable modification of clinical symptoms of OA, especially knee OA.

Author's contribution

Takayuki Okumo: Contribute to cell culture and sample collection

Yusuke Ohashi and Midori Tanaka: Contribute to assay for adiponectin mRNA expression and Akt phosphorylation.

Masataka Sunagawa: Contribute to statistical analysis of data, Contribute to preparation of Figures (Figure 1 to Figure 4). Contribute to final check of the entire manuscript.

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

Competing Interests

The authors declare that they have no competing interests.

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