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Recent aspects of the anti-inflammatory actions of glucosamine

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ABSTRACT

Glucosamine, a naturally occurring amino monosaccharide, is present in the connective and cartilage tissues as a component of glycosaminoglycans. Thus, glucosamine has been widely used to treat osteoarthritis, a joint disease characterized by cartilage degeneration, in humans. In addition, glucosamine is expected to exert an anti-inflammatory action, since glucosamine suppresses inflammatory cell activation. To further extend the anti-inflammatory actions of glucosamine, we investigated the effects of glucosamine on synovial cells, endothelial cells and intestinal epithelial cells using *in vitro* and *in vivo* systems. Firstly, glucosamine suppressed the IL-1β-induced activation of synovial cells *in vitro*. Furthermore, glucosamine administration repressed synovial cell hyperplasia, cartilage destruction and inflammatory cell infiltration in rat adjuvant arthritis. Secondary, glucosamine suppressed the TNF-α-induced activation of intestinal epithelial cell HT-29 *in vitro*. In addition, glucosamine administration improved the clinical symptoms, and colonic inflammation and tissue injury in dextran sulfate sodium-induced colitis in rats. Finally, glucosamine suppressed the TNF-α-induced activation of endothelial cells *in vitro*. Moreover, glucosamine administration repressed the formation of atherosclerotic lesion and infiltration of inflammatory cells into the lesion in spontaneously hyperlipidemic mice B6 KOR *Apo*^{shl}. Together these observations support the idea that glucosamine can function as not only a chondroprotective agent but also an anti-inflammatory molecule in the body.

1. Introduction

Glucosamine, a naturally occurring amino monosaccharide, is present in the connective and cartilage tissues as a component of glycosaminoglycans. Thus, glucosamine has been widely used to treat osteoarthritis, a joint disease characterized by cartilage degeneration, in humans (Crolle & D'Este, 1980). In fact, glucosamine normalizes cartilage metabolism, so as to stimulate the synthesis and inhibit the degradation of glycosaminoglycans, and restores articular function (Fenton, Chlebek-Brown, Peters, Caron, & Orth, 2000; Oegema, Deloria, Sandy, & Hart, 2002). In addition, glucosamine is expected to exert an anti-inflammatory action, since glucosamine suppresses inflammatory cell activation (such as neutrophils, cytotoxic T-lymphocytes and dendritic cells) (Hua, Sakamoto, & Nagaoka, 2002; Ma et al., 2002; Zhang, Yu, Gran, & Rostami, 2005). In this paper, to further extend our hypothesis on the anti-inflammatory actions of glucosamine, we investigated the effects of glucosamine on the activation of synovial cells, endothelial cells and intestinal epithelial cells using *in vitro* and *in vivo* systems (Hua, Suguro, Hirano, Sakamoto, & Nagaoka, 2005; Hua et al., 2007; Ju, Hua, Sakamoto, Ogawa, & Nagaoka, 2008; Yomogida, Hua, Sakamoto & Nagaoka, 2008; Yomogida, Kojima, et al., 2008; Yomogida, Kojima, Ju, Sakamoto & Nagaoka, 2008).

2. Experimental

2.1. Synovial cell culture

Synovial tissues were obtained from patients with rheumatoid arthritis undergoing total joint replacement at Juntendo University Hospital. Fibroblast-like synovial cells (purity > 95%) were isolated form synovial tissues and used (Hua et al., 2007).

Synovial cells were stimulated with 4 ng/ml IL-1β in the presence of 0.01–1 mM glucosamine hydrochloride (Koyo Chemical) in RPMI 1640 medium containing 10% fetal calf serum (FCS) for 24 h at 37 °C. IL-8 in the culture supernatants was quantified by ELISA (eBioscience). For quantification of nitric oxide and PG (prostaglandin) E_2, synovial cells were stimulated with IL-1β in the presence of glucosamine in RPMI 1640 for 24 h at 37 °C. Nitric oxide was measured as nitrite accumulation in the culture supernatants by Griess assay (Cayman Chemical), and PGE_2 was measured by ELISA (Cayman Chemical).

For analysis of p38 MAPK phosphorylation, a primary human synovial cell line CS-ABI-479 (Dainippon Pharmaceutical) was used.

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CS-ABI-479 cells were stimulated with IL-1β in the presence of glucosamine in CS-C complete medium for 5 min at 37 °C. Thereafter, the cells were harvested in lysis buffer, and subjected to 10% SDS-PAGE for detection of phosphorylated p38 MAPK by western blotting using mouse anti-phosphorylated p38 MAPK monoclonal antibody (BD Bioscience Pharmingen).

2.2. Adjuvant arthritis model in rats

Adjuvant arthritis was induced in 8-week-old male Wistar rats by a single intradermal injection of Freund's complete adjuvant (FCA) (Hua et al., 2005). Glucosamine hydrochloride dissolved in distilled water (60 mg/ml) was administered orally once a day for 22 days by gavage in a dose of 300 or 1000 mg/kg. Five animals were employed in each experimental group (naive control rats and FCA-injected rats without or with glucosamine administration).

Progression of adjuvant arthritis was clinically evaluated for their characteristic signs and symptoms by employing an arthritis score, based on erythema and swelling of the joint, and incapability to bend the ankle. Animals were sacrificed on day 22, and the FCA-injected right and -uninjected left legs were resected. Tissue sections (10 μm) were stained with hematoxylin/eosin, and articular lesions were observed under a light microscopy.

Blood samples were collected from abdominal aorta on day 22, and plasma nitric oxide (nitrite and nitrate) and PGE_2 were measured by a nitrate/nitrite colorimetric assay kit (BioDynamics Laboratory) and ELISA (Cayman Chemical), respectively.

2.3. Colonic epithelial cell culture

A human colonic epithelial cell line HT-29 was utilized (American Type Culture Collection) (Yomogida, Hua, et al., 2008). Cells were stimulated with 10 ng/ml TNF-α in the presence of 0.1–10 mM glucosamine hydrochloride for 12 h at 37 °C in McCoy's 5A medium containing 1% FCS. IL-8 in the culture supernatants was quantified by ELISA (R&D Systems).

HT29 cells were harvested in lysis buffer after 12-h stimulation, and subjected to 10% SDS-PAGE for detection of intercellular adhesion molecule (ICAM)-1 by western blotting using rabbit anti-human ICAM-1 antibody (Santa Cruz). Furthermore, HT29 cells were harvested in lysis buffer after 10-min stimulation, and subjected to SDS-PAGE for detection of phosphorylated p38 MAPK and NF-κB p65 by western blotting using mouse anti-phosphorylated p38 MAPK monoclonal antibody and rabbit anti-phosphorylated NF-κB p65 antibody (Cell Signaling Technology), respectively.

2.4. Dextran sulfate sodium (DSS)-induced colitis model in rats

To elicit colitis, male Sprague–Dawley rats weighing 280–310 g were administered with 4% DSS *ad libitum* for 7 days, followed by administration with distilled water for 7 days, and further administered with DSS for 11 days ($n = 8$) (Yomogida, Kojima, et al., 2008). In addition, rats were administered with glucosamine hydrochloride (10 mg/ml) dissolved in 4% DSS (+GlcN, $n = 8$), or distilled water (control, $n = 7$) *ad libitum* for the experimental period. Animals received an average of 40 ml glucosamine solution (400 mg glucosamine/animal/day).

Colitis was evaluated using a disease activity index (DAI), using parameters of body-weight loss, stool consistency and bleeding. Rats were sacrificed on day 25, and the length and weight of the colon were measured. Tissue sections (3 μm) were stained with hematoxylin/eosin, and histopathologically scored, based on the inflammatory cell infiltration, shortening of crypt and destruction of crypt. Furthermore, phosphorylation of NF-κB was immunohistochemically detected in colon tissues using rabbit anti-phosphorylated NF-κB p65 antibody.

Blood samples were collected on day 25, and serum cytokines TNF-α, IL-6 and cytokine-induced neutrophil chemoattractant factor 1 (CINC-1, CXC chemokine family) were quantified by ELISA (eBioscience and R&D Systems).

2.5. Endothelial cell culture

Human umbilical vein endothelial cells (HUVEC; Sanko Junyaku) were stimulated with 0.5 ng/ml TNF-α in the presence or absence of 0.1–10 mM glucosamine for 24 h at 37 °C in EGM-2 medium (Ju et al., 2008). In some experiments, HUVEC were preincubated with 0.5 mM alloxan for 2 h prior to the stimulation. After the stimulation, the supernatants were recovered and monocyte chemoattractant protein (MCP)-1 was quantified by ELISA (R&D system).

Furthermore, the cells were harvested in lysis buffer, and subjected to 10% SDS-PAGE for detection of ICAM-1, and phosphorylated p38MAPK and NF-κB by western blotting using rabbit anti-human ICAM-1 antibody, mouse anti-phosphorylated p38MAPK monoclonal antibody and rabbit anti-phosphorylated NF-κB p65 antibody, respectively. Moreover, *O*-N-acetylglucosamine (GlcNAc)-modified proteins were detected by western blotting using mouse anti-*O*-GlcNAc monoclonal antibody (Covance).

2.6. Atherosclerosis model in mice

Spontaneously hyperlipidemic B6.KOR-*Apoe*^{shl} mice were used as a model of atherosclerosis (Matsushima et al., 2001; Yomogida, Kojima, Ju, et al., 2008). B6.KOR-*Apoe*^{shl} mice (8-week old) were orally administered with distilled water or glucosamine hydrochloride (300 or 1000 mg/kg) once a day for 3 months by gavage; 10 mice were employed in each experimental group (without or with glucosamine administration). Furthermore, C57BL/6 mice were used as an unatherosclerotic control.

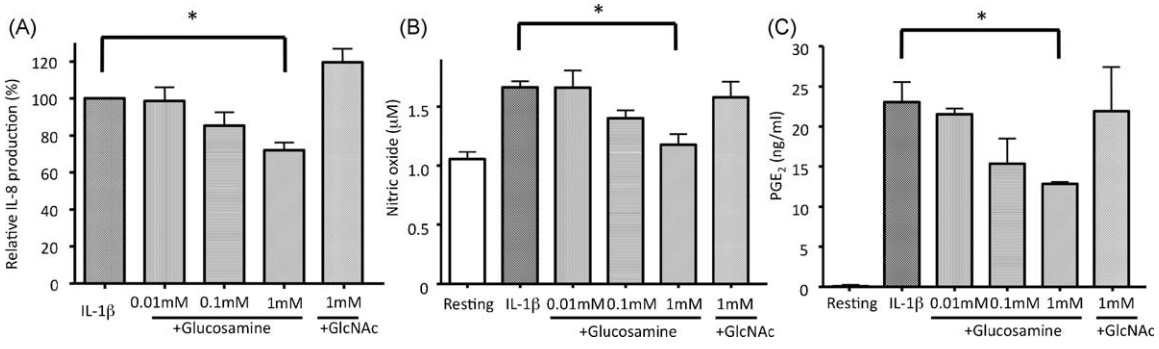

Fig. 1. Effect of glucosamine and N-acetylglucosamine (GlcNAc) on IL-8 (A), nitric oxide (B) and PGE_2 (C) production by synovial cells. $^*P < 0.05$.

After 3 months, animals were sacrificed, and blood samples were collected from inferior vena cava for measuring serum total cholesterol, HDL-cholesterol and lipid peroxide using commercially available kits (Wako Pure Chemical Industries and Kyowa Medex). Thereafter, hearts were perfused with PBS and 10% buffered formalin, and resected. Hearts were stained with Oil Red O for evaluating lipid accumulation in the aortic bulb. Moreover, tissue sections of aortic bulb (3 μm) were stained with hematoxylin/eosin, and infiltrated inflammatory cells were counted under a light microscopy.

2.7. Statistical analysis

Data are expressed as mean ± SD or SEM, and analyzed for significant difference by a one-way analysis of variance (ANOVA) with multiple comparison test, or Student's *t*-test (Prism 4). Differences were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. In vitro effect of glucosamine on synovial cell activation

First, we examined the effect of glucosamine on synovial cell activation *in vitro* (Hua et al., 2007). As shown in Fig. 1A–C, glucosamine dose-dependently inhibited the IL-1β-induced production of IL-8, nitric oxide and PGE_2 by synovial cells. In contrast, N-acetylglucosamine, an analogue of glucosamine could not affect the production of these mediators even at 1 mM.

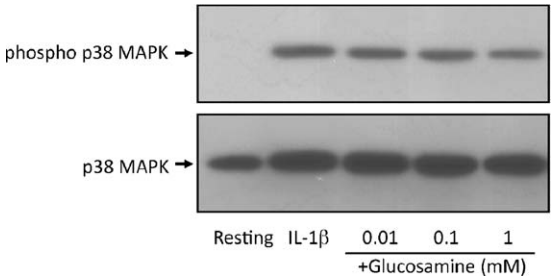

Fig. 2. Effect of glucosamine on the IL-1β-induced phosphorylation of p38 MAPK in synovial cells.

Furthermore, we investigated the effect of glucosamine on the activation of p38 MAPK, a signaling molecule involved in the synovial cell activation. Consistent with its suppressive action on the mediators, glucosamine inhibited the IL-1β-induced phosphorylation of p38 MAPK (Fig. 2).

3.2. In vivo effect of glucosamine on adjuvant arthritis in rats

Next, we investigated the effect of glucosamine on the synovial cell activation *in vivo*, using rat adjuvant arthritis, a model of rheumatoid arthritis (Hua et al., 2005). Of note, glucosamine administration significantly suppressed the increase in arthritis score (Fig. 3A). Moreover, histopathological examination indicated that glucosamine administration strongly suppressed the

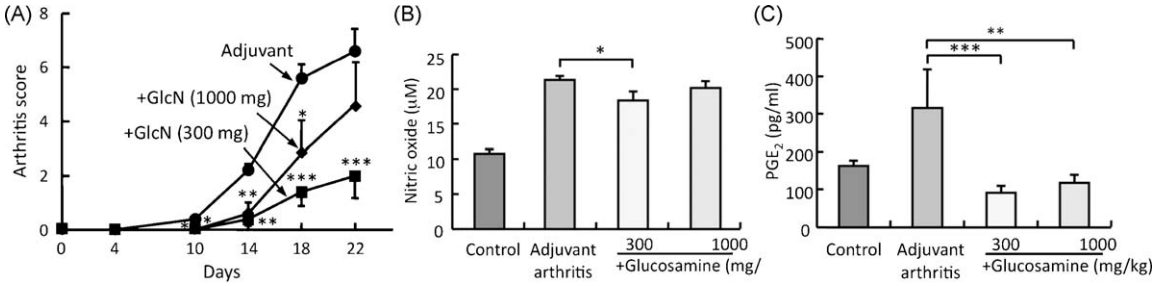

Fig. 3. Effect of glucosamine (GlcN) on arthritis score (A), nitric oxide (B) and PGE_2 (C) production in adjuvant arthritis rats. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

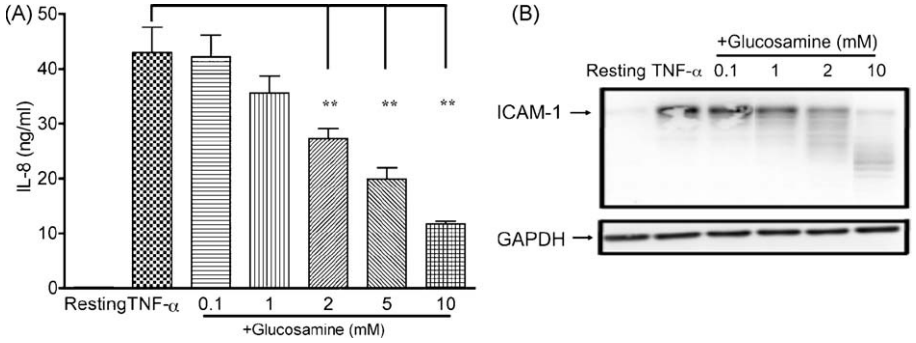

Fig. 4. Effect of glucosamine on IL-8 production (A) and ICAM-1 expression (B) by HT-29 cells. $^{**}P < 0.01$.

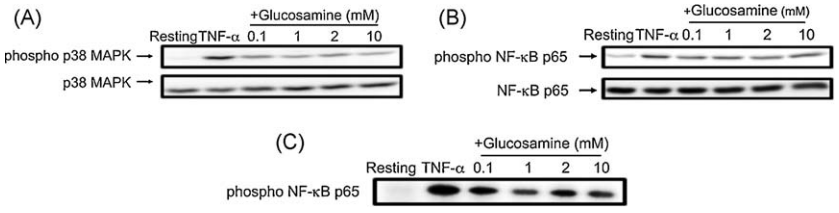

Fig. 5. Effect of glucosamine on the phosphorylation of p38 MAPK (A) and NF-κB (B), and the nuclear translocation of phosphorylated NF-κB (C) in HT-29 cells.

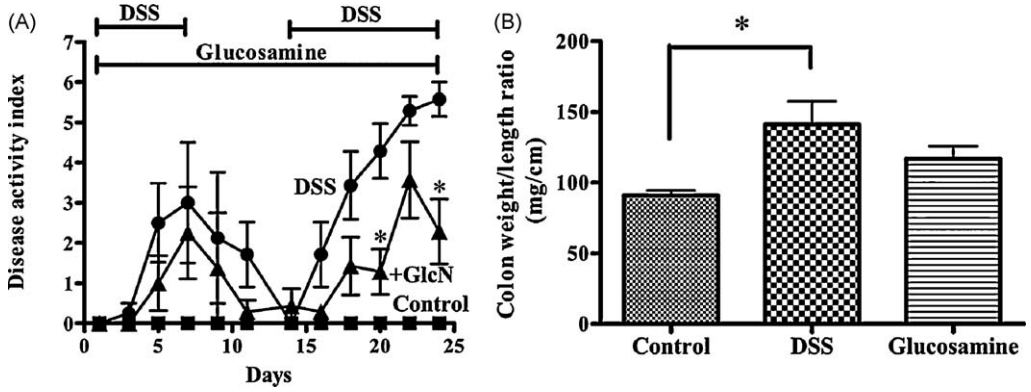

Fig. 6. Effects of glucosamine (GlcN) on disease activity index (A) and colon weight/length ratio (B) in DSS-induced colitis. $^{*}P < 0.05$.

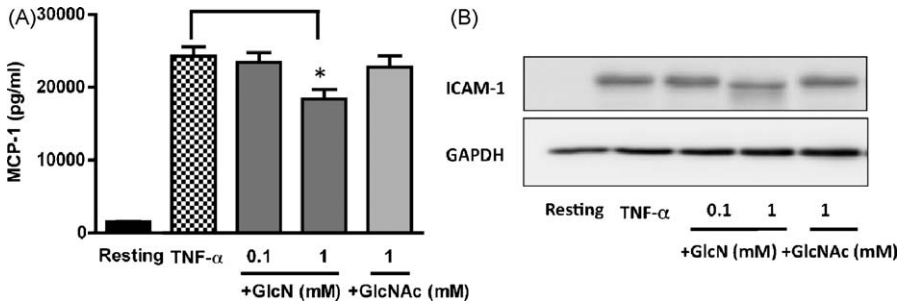

Fig. 7. Effect of glucosamine and GlcNAc on MCP-1 production (A) and ICAM-1 expression (B) by HUVEC. $^{*}P < 0.05$.

synovial hyperplasia, pannus formation with cartilage erosion, and severe leukocyte infiltration (mononuclear cells and neutrophils) in the knee joints of FCA-injected right hind paws (data not shown). In addition, glucosamine administration significantly reduced the plasma levels of nitric oxide and PGE_2 in adjuvant arthritis rats (Fig. 3B and C). Thus, glucosamine relieved the clinical symptoms of adjuvant arthritis *in vivo*, accompanied with the suppression of synovial cell activation and production of inflammatory mediators.

3.3. *In vitro* effect of glucosamine on intestinal cell activation

To further investigate the effects of glucosamine on inflammatory bowel diseases (IBD), we first evaluated the effect of glucosamine on the intestinal epithelial cell activation *in vitro*, using a human colonic epithelial cell line HT-29 (Yomogida, Hua, et al., 2008). Importantly, glucosamine dose-dependently suppressed the TNF-α-induced IL-8 production and ICAM-1 expression by HT-29 cells (Fig. 4A and B). Moreover, glucosamine dose-dependently suppressed the phosphorylation of p38 MAPK and NF-κB as well as the nuclear translocation of phosphorylated NF-κB p65 (Fig. 5A–C).

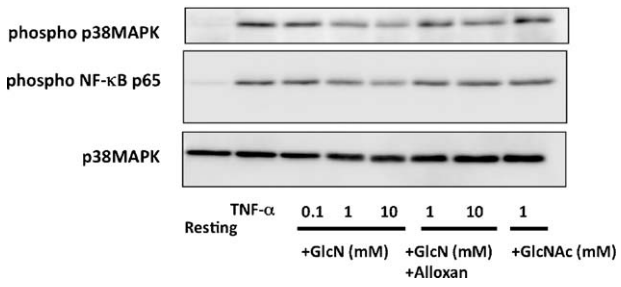

Fig. 8. Effect of glucosamine and GlcNAc on the phosphorylation of p38 MAPK and NF-κB in HUVEC. $^{*}P < 0.05$.

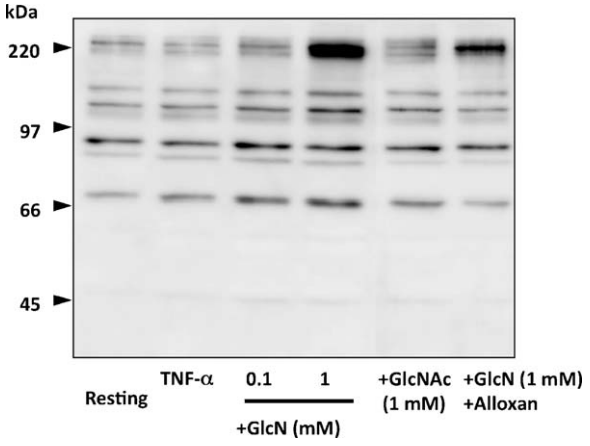

Fig. 9. Glucosamine-induced *O*-GlcNAc modification in HUVEC.

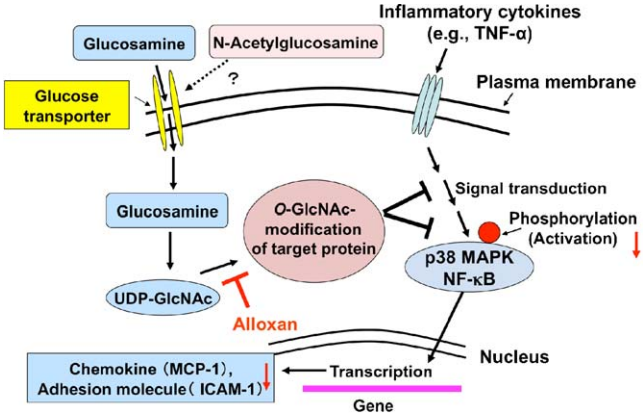

Fig. 10. Possible mechanism for the *O*-GlcNAc modification-mediated suppression of endothelial cell activation by glucosamine.

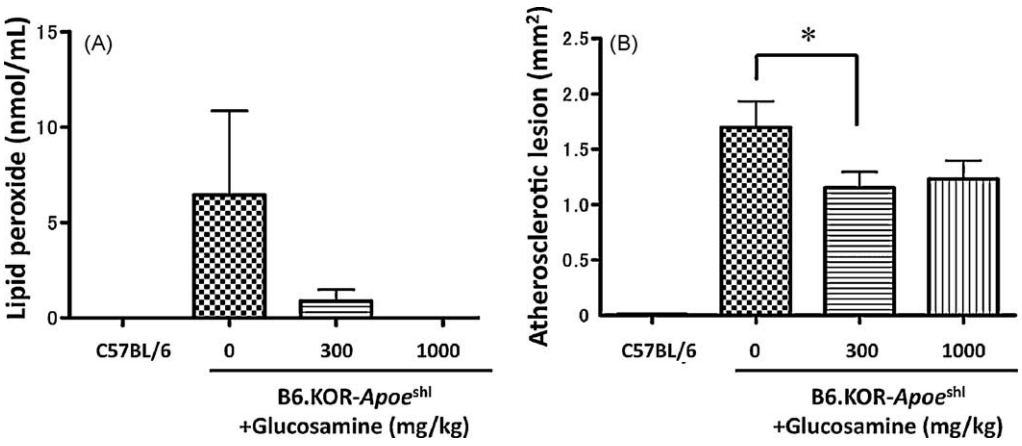

Fig. 11. Effect of glucosamine on the lipid peroxide level (A) and atherosclerotic lesion (B) in B6.KOR-*Apoe*^{shl} mice. $^{*}P < 0.05$.

Thus, glucosamine likely inhibits the TNF-α-induced activation of intestinal epithelial cells (IL-8 production and ICAM-1 expression) via the suppression of signaling molecules such as p38MAPK and NF-κB.

3.4. In vivo effect of glucosamine on DSS-induced colitis in rats

Next, we evaluated the effect of glucosamine on IBD *in vivo*, using dextran sulfate sodium (DSS)-induced colitis in rats, a model of IBD (Yomogida, Kojima, et al., 2008). Of note, glucosamine administration improved the clinical symptom (evaluated by disease activity index) (Fig. 6A), and suppressed colonic inflammation (evaluated by colon weight/length ratio) (Fig. 6B) and tissue injury (erosion, ulcer and destruction of crypt) (data not shown) in DSS-induced colitis. Furthermore, glucosamine inhibited the production of CINC-1, and the activation of intestinal epithelial cells (as evidenced by the phosphorylation of NF-κB) in DSS-induced colitis (data not shown).

These observations suggest that glucosamine suppresses the activation of intestinal epithelial cells *in vivo*, thereby exhibiting anti-inflammatory action on IBD.

3.5. In vitro effect of glucosamine on endothelial cell activation

Atherosclerosis is now recognized as a chronic inflammatory disease. Finally to investigate the effects of glucosamine on atherosclerosis, we determined the effect of glucosamine on the endothelial cell activation *in vitro*, using human umbilical vein endothelial cells (HUVEC) (Ju et al., 2008). The results indicated that glucosamine but not N-acetylglucosamine suppressed the TNF-α-induced production of MCP-1 and expression of ICAM-1 (Fig. 7A and B), and abrogated the phosphorylation of p38MAPK and NF-κB (Fig. 8) in HUVEC.

Further, we investigated whether glucosamine induces *O*-GlcNAc modification, which plays a role in the modulation of cell functions, including cell signaling (Wells, Vosseller, & Hart, 2001; Hanover, 2001). Of note, glucosamine but not N-acetylglucosamine induced the *O*-GlcNAc modification in HUVEC, which was prevented by alloxan, an *O*-GlcNAc modification inhibitor (Fig. 9).

Moreover, the levels of *O*-GlcNAc modification were negatively correlated with the production of MCP-1 and expression of ICAM-1 (data not shown), and phosphorylation of p38MAPK and NF-κB (Fig. 8). Thus, glucosamine is likely to inhibit the endothelial cell activation (TNF-α-induced ICAM-1 production and MCP-1 expression) by suppressing p38 MAPK and NF-κB signaling via the *O*-GlcNAc modification (Fig. 10).

3.6. In vivo effect of glucosamine on atherosclerosis in mice

Lastly, we investigated the effect of glucosamine on atherosclerosis *in vivo*, using spontaneously hyperlipidemic B6.KOR-*Apoe*^{shl} mice as a model of atherosclerosis (Yomogida, Kojima, Ju, et al., 2008). The results indicated that glucosamine administration significantly lowered the serum level of lipid peroxide (Fig. 11A), which is involved in the pathogenesis of atherosclerosis. In contrast, glucosamine administration did not affect the serum levels of total cholesterol and HDL-cholesterol (data not shown). Furthermore, glucosamine administration suppressed the formation of atherosclerotic lesion (lipid accumulation) (as evidenced by Oil O Red stain; Fig. 11B) and infiltration of inflammatory cells into the lesion (data not shown). Thus, glucosamine is likely to suppress lipid peroxide formation, thereby possibly exhibiting anti-atherosclerotic action *in vivo*.

Together these observations support our idea that glucosamine can function as not only a chondroprotective agent but also an anti-inflammatory molecule in the body. The *in vivo* actions of glucosamine as an anti-inflammatory agent against inflammatory articular disorders, IBD and atherosclerosis should be carefully evaluated in humans in the future.

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