Glucosamine, a naturally occurring amino monosaccharide modulates LL-37-induced endothelial cell activation

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Abstract. Atheroscleros is now considered as a chronic inflammatory disease, and glucosamine has a potential to exhibit anti-inflammatory action. Thus, we investigated the effect of glucosamine on LL-37-induced endothelial cell activation. HUVEC (human umbilical vein endothelial cells) were stimulated by LL-37 in the presence or absence of glucosamine (0.01-1 mM) or its analogue, N-acetylglucosamine (0.1-1 mM). mRNA expression of MCP-1 (monocyte chemoattractant protein-1) and ICAM-1 (intercellular adhesion molecule-1) was evaluated by real-time RT-PCR, and their protein levels were analyzed by ELISA and Western blotting, respectively. Furthermore, the effect of glucosamine on O-N-acetylglucosamine (O-GlcNAc) modification was evaluated by Western blotting. Glucosamine but not N-acetylglucosamine suppressed the LL-37-induced expression of MCP-1 and ICAM-1 at both mRNA ($p<0.05$ at 0.1 mM) and protein levels ($p<0.05$ at 1 mM). Of interest, O-GlcNAc modification was induced by incubating HUVEC with glucosamine ($p<0.05$ at 1 mM) but not N-acetylglucosamine. Of note, alloxan, an O-N-acetylglucosamine transferase inhibitor, which prevented the glucosamine-induced O-GlcNAc modification, abrogated the suppressive effect of glucosamine on MCP-1 and ICAM-1 expression ($p<0.05$ at 0.5 mM). These observations suggest that glucosamine modulates endothelial cell activation possibly via O-GlcNAc modification, and may exhibit an anti-inflammatory action on atherosclerosis.

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Introduction

In recent years many different clinical and pathological studies have altered the concept of pathogenesis of atherosclerosis. Although large lipid deposits can be seen in atheromatous lesions and the role of different lipoproteins are suggested in the pathogenesis of the disease, atherosclerosis is no longer considered to be a primary disorder of lipid accumulation (1). It is a state of disordered immunity in which there is dynamic interaction between endothelial dysfunction, inflammation and repeated cycles of 'wound healing response' (2). Expression of endothelial cell adhesion molecules such as intercellular adhesion molecule (ICAM-1), induces the binding of monocytes and T lymphocytes, thus initiating an inflammatory process that ultimately leads to the formation of atherosclerotic plaque (3). The migration of inflammatory cells into the subendothelial space is facilitated by chemoattractants such as monocyte chemoattractant protein-1 (MCP-1) (4) and oxidized low-density lipoprotein (5). Once migrated into the subendothelial space, monocytes mature into macrophages and express scavenger receptors to internalize modified lipoproteins, which gives rise to lipid-laden macrophages or foam cells (6).

In mammals at least two distinct families of antimicrobial peptides (AMPs) are identified. Defensins form a group with most representatives, and cathelicidin-derived antimicrobial peptides form a second group of AMPs. They are detected in blood, secretions, epithelial tissues and neutrophil granules, and exhibit potent antimicrobial activities against both Gram-positive and -negative bacteria, fungi and certain viruses (7). Although many cathelicidin members have been isolated from mammals, only one cathelicidin has been identified in humans and named human cationic antibacterial protein of 18 kDa (hCAP18) (8). The mature antibacterial peptide, LL-37 owes its name from its 37-amino acid peptide with the two leading leucine residues. LL-37 was first detected in neutrophils, but later shown to be expressed in various epithelial cells, and specific lymphocyte and monocyte populations (9). LL-37 not only kill bacteria but also binds to LPS and blunt its biological activities (10). Furthermore, LL-37 possesses the ability to chemoattract immune and inflammatory cells, including neutrophils, monocytes and

T lymphocytes via the action on a low affinity formyl-peptide receptor-like 1 (FPRL1) (11). In addition, LL-37 suppresses neutrophil apoptosis via the activation of FPRL1 and $P2X_7$, a nucleotide receptor (12). Of importance, it has been recently revealed that LL-37 is expressed in the atherosclerotic plaques and involved in inflammatory responses in endothelial cells via the induction of ICAM-1 and MCP-1 expression, and vascular smooth muscle cell death (13,14).

Glucosamine, a naturally occurring amino monosaccharide, is acting as a preferred substrate for biosynthesis of glyco-saminoglycan, and is used for the treatment of osteoarthritis more than two decades in Europe (15). Several short- and long-term clinical trials in osteoarthritis have shown the significant symptom-modifying effect of glucosamine with no side effects (16). Furthermore, glucosamine is shown to inhibit the expression of inducible nitric oxide synthase in macrophages (17), and neutrophil functions such as superoxide generation, phagocytosis, granule enzyme release, chemotaxis and CD11b expression (18), thereby possibly exhibiting anti-inflammatory action. More recently, glucosamine has been revealed to inhibit ICAM-1 expression in human retinal pigment epithelial cells, which suggests the potential of glucosamine to attenuate inflammation in eyes (19). Based on these findings, we hypothesized that glucosamine may affect the endothelial cell activation. In this study, we investigated the effect of glucosamine on the activation of endothelial cells (expression of a monocyte chemoattractant factor MCP-1 and an adhesion molecule ICAM-1) induced by LL-37, an AMP expressed in the atherosclerotic lesions.

Materials and methods

Reagents. D-Glucosamine hydrochloride was supplied from Koyo Chemical Co. (Tokyo, Japan). N-Acetyl-D-glucosamine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Alloxan monohydrate was purchased from Sigma Chemical Co. (St. Louis, MO). A 37-mer peptide of hCAP18 (LL-37; L1LGDFFRKSKEKIGKEFKRIVQRIKDF LRNLVPRTES[37]) was synthesized and purified, as previously described (10).

Endothelial cells. Human umbilical vein endothelial cells (HUVEC) and endothelial cell medium EGM-2 were purchased from Sanko Junyaku Co. (Tokyo, Japan). HUVECs were maintained in EGM-2 medium at 37˚C in a CO_2 incubator.

Analysis of MCP-1 and ICAM-1 mRNA expression by real-time RT-PCR. HUVEC were plated at $1.5x10^5$/well in 6-well plates in EGM-2 medium for 24 h at 37˚C (50-60% confluent). The HUVEC were incubated in the presence or absence of 0.01-1 mM glucosamine or 0.1-1 mM N-acetylglucosamine for 2 h, and then stimulated with 4 μM LL-37 for 24 h at 37˚C. Thereafter, the total RNA was purified using an RNeasy Plus mini kit (Qiagen, Valencia, CA), and first-strand cDNA was synthesized with total RNA (1 μg), a random primer and ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) at 42˚C for 20 min, followed by termination at 99˚C for 5 min. Further, a quantitative real-time PCR was performed using

7500 Real-Time PCR system (Applied Biosystems, Warrington, UK). Reactions were carried out in a reaction mixture consisting of 25 μl solution containing power SYBR Green PCR master mix (Applied Biosystems) and 200 nM of each primer (ICAM-1, MCP-1 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) forward or reverse primer; Takara, Tokyo, Japan).

Analysis of MCP-1 protein expression by ELISA. HUVEC (50-60% confluent in 24-well plates) were incubated in the presence or absence of 0.1-1 mM glucosamine or 0.1-1 mM N-acetylglucosamine for 2 h, and then stimulated with 4 μM LL-37 for 24 h at 37˚C. The supernatants were removed and used for the quantification of MCP-1 protein using a Duoset MCP-1 ELISA development system (R&D System, UK). In some experiments, HUVEC were preincubated with 0.5 mM alloxan for 2 h and further incubated with 1 mM glucosamine for 2 h, before stimulation with 4 μM LL-37.

Analysis of ICAM-1 protein expression by Western blotting. HUVEC (50-60% confluent in 6-well plates) were incubated in the presence or absence of 0.1-1 mM glucosamine or 0.1-1 mM N-acetylglucosamine for 2 h, and then stimulated with 4 μM LL-37 for 24 h at 37˚C. After washing with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), the cells were recovered in 250 μl of lysis buffer (10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 100 mM NaCl, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA and 1 mM di-isopropyl fluorophosphate) containing 1/25 v/v Complete™ (Roche Diagnostics, Mannheim, Germany). Lysates were placed on ice for 30 min, and centrifuged at 14,000 x g for 10 min. The supernatants were recovered, and samples (10 μg protein/lane) were subjected to 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Separated proteins were transferred to polyvinylidene difluoride membrane (Millipore Corporation, Bedford, MA), and the membrane was blocked in 5% skim milk and probed with rabbit anti-human ICAM-1 polyclonal antibody (H-108; Santa Cruz Biotechnology, Santa Cruz, CA). After washing with PBS-0.05% Tween, the membrane was further probed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Chemicon International, Temecula, CA), and ICAM-1 was finally detected with a chemiluminescent substrate (Pierce, Rockford, IL). The detected bands were quantified using LAS-3000 image analyzer (Fujifilm Corporation, Tokyo, Japan).

Thereafter, the antibodies were stripped from the membrane using Restore Western stripping buffer (Pierce). GAPDH contained in each sample were detected with mouse anti-GAPDH monoclonal antibody (MAB374, Chemicon International) and HRP-conjugated goat anti-mouse IgG/IgM (Chemicon International). The protein contents were deter-mined with a BCA protein assay kit (Pierce).

Detection of O-N-acetylglucosamine modification by Western blotting. HUVEC (50-60% confluent in 6-well plates) were incubated in the presence or absence of 1 mM glucosamine or 1 mM N-acetylglucosamine for 2 h, and then stimulated with 4 μM LL-37 for 24 h at 37˚C. Alternatively, HUVEC were preincubated with 0.5 mM alloxan for 2 h and further
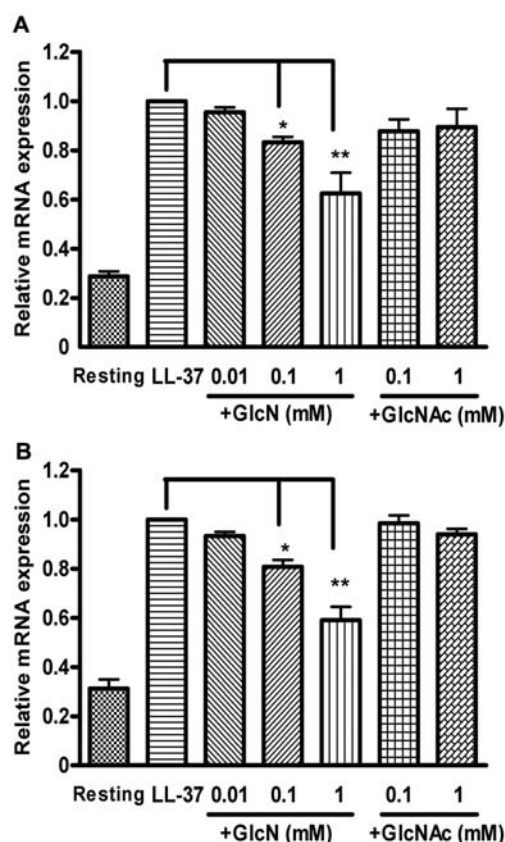
Figure 1. Effect of glucosamine on LL-37-induced MCP-1 and ICAM-1 mRNA expression in HUVEC. HUVEC were incubated without (Resting) or with 4 μM LL-37 (LL-37) in the absence or presence of 0.01-1 mM glucosamine (GlcN) or N-acetylglucosamine (GlcNAc). MCP-1, ICAM-1 and GAPDH (a loading control) mRNA was detected by quantitative real-time RT-PCR. MCP-1 (A) and ICAM-1 (B) mRNA expression was expressed as a ratio relative to LL-37-stimulation alone. Data are mean ± SEM from 4 independent experiments; *p<0.05, **p<0.01.
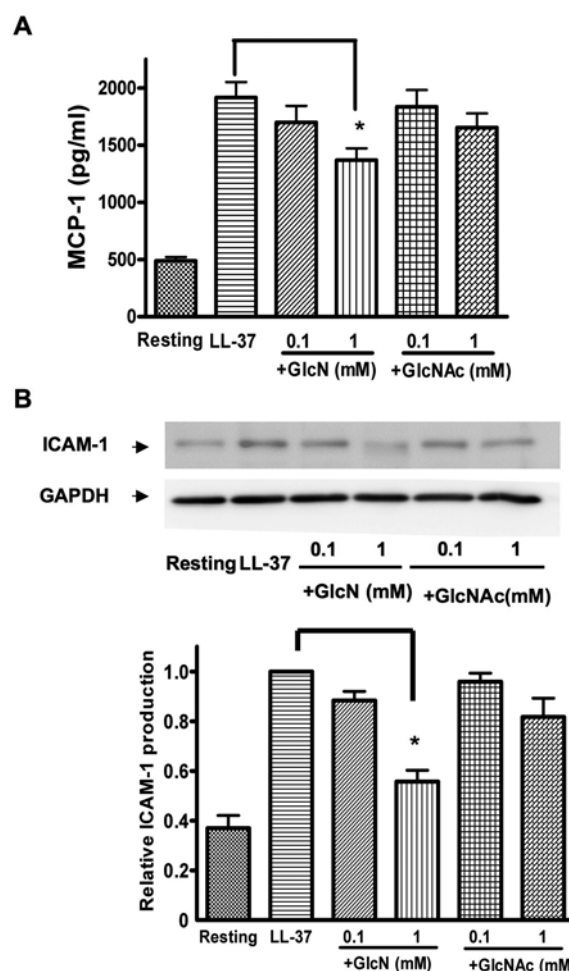

Figure 2. Effect of glucosamine on MCP-1 and ICAM-1 expression in HUVEC. HUVEC were incubated without (Resting) or with 4 μM LL-37 (LL-37) in the absence or presence of 0.1-1 mM glucosamine (GlcN) or N-acetylglucosamine (GlcNAc). The supernatants and cell lysates were recovered, and MCP-1 (A) and ICAM-1 (B) were quantitated by ELISA and Western blotting, respectively. ICAM-1 level was expressed as a ratio of LL-37-stimulation. Data are mean ± SEM from 6 independent experiments for both MCP-1 and ICAM-1; *p<0.05.

incubated with 1 mM glucosamine for 2 h before 4 μM LL-37-stimulation. Samples (15 μg protein/lane) were applied to 7.5% SDS-PAGE, and the blotted membrane was immersed in 100% methanol and dried at room temperature. Then, the membrane was probed with mouse anti-O-GlcNAc (ß-O-linked N-acetylglucosamine) monoclonal antibody (CTD110.6; Covance, Berkeley, CA) in 1% casein/PBS, and further probed with HRP-conjugated goat anti-mouse IgG/IgM (Chemicon International) in 1% casein/PBS. The O-N-acetylglucosamine-modified proteins were detected, and analyzed, as described above.

Statistical analysis. Data are expressed as mean ± 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, GraphPad Software, San Diego, CA). Differences were considered statistically significant at p<0.05.

Results

Effect of glucosamine on LL-37-induced expression of MCP-1 and ICAM-1 mRNA. To evaluate the effect of glucosamine on the MCP-1 and ICAM-1 mRNA expression, we stimulated HUVEC with LL-37, an AMP expressed in the athero-sclerotic plaques, in the absence or the presence of glucosamine or N-acetylglucosamine. As shown in Fig. 1, glucosamine dose-dependently inhibited the LL-37-induced expression of MCP-1 and ICAM-1 mRNA detected by real-time RT-PCR (p<0.05 at 0.1 and 1 mM glucosamine). In contrast, N-acetylglucosamine, an analogue of glucosamine, did not significantly affect the mRNA expression.

Effect of glucosamine on LL-37-induced expression of MCP-1 and ICAM-1 proteins. Since glucosamine inhibited the MCP-1 and ICAM-1 mRNA expression (Fig. 1), we next examined the effect of glucosamine on the LL-37-induced expression of MCP-1 and ICAM-1 proteins. Consistent with its suppressive effect on the mRNA expression, glucosamine inhibited the protein expression of MCP and ICAM-1 in a dose-dependent manner (p<0.05 at 1 mM glucosamine) (Fig. 2). As expected, N-acetylglucosamine gave no substantial effect on the protein expression.
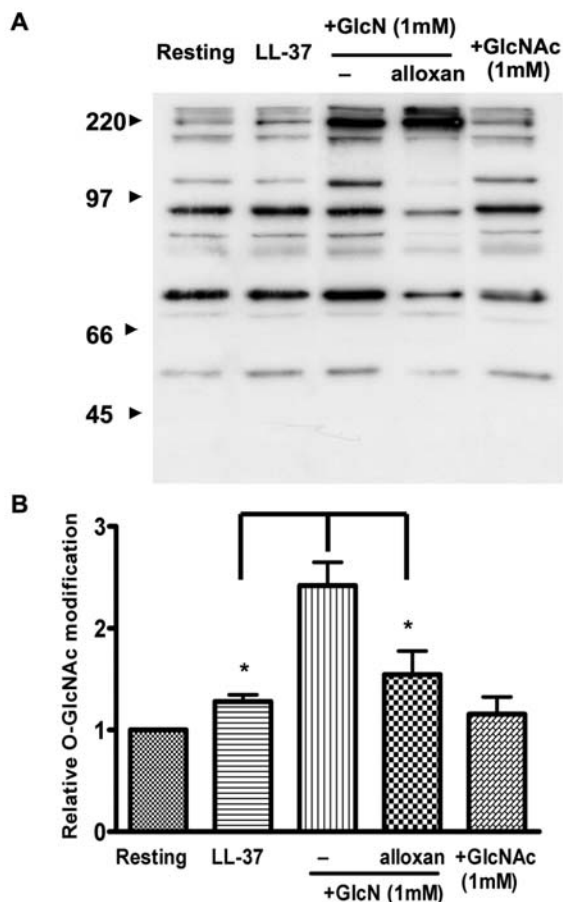
A

B

Figure 3. Glucosamine-induced O-GlcNAc modification in HUVEC. HUVEC were incubated without (Resting) or with 4 μM LL-37 (LL-37) in the absence or presence of 1 mM glucosamine (GlcN) or N-acetylglucosamine (GlcNAc). Alternatively, HUVEC were stimulated with 4 μM LL-37 in the presence of glucosamine and alloxan (0.5 mM). O-GlcNAc-modified proteins were evaluated by Western blotting (A). All the detected bands were quantified and summed up. O-GlcNAc modification levels were expressed as a ratio relative to Resting cells (B). Data are mean ± SEM from 3 independent experiments; *p<0.05.

Effect of glucosamine on O-GlcNAc modification. Modification of cellular proteins with *O*-linked-N-acetylglucosamine is involved in the modulation of cell functions, such as transcription and translation (20). Thus, we asked whether glucosamine induces *O*-GlcNAc modification, which accounts for the suppressive effect of glucosamine on endothelial cell activation. Preliminarily, we confirmed that glucosamine but not N-acetylglucosamine dose-dependently induced the protein *O*-GlcNAc modification detected by Western blotting (0.1, 1 and 10 mM) (data not shown). When the cells were stimulated with LL-37 in the presence of 1 mM glucosamine, *O*-GlcNAc modification was observed (p<0.05), although LL-37 alone did not induce *O*-GlcNAc modification (Fig. 3). Of interest, alloxan, an inhibitor of *O*-GlcNAc modification, effectively prevented the glucosamine-induced *O*-GlcNAc modification (p<0.05) (Fig. 3). As expected, N-acetylglucosamine did not essentially induce *O*-GlcNAc modification.

Effect of alloxan on the glucosamine-induced suppression of MCP-1 and ICAM-1 protein expression. Glucosamine inhibited MCP-1 and ICAM-1 expression but N-acetylglucosamine did not. Similarly, glucosamine but not N-actylglucosamine
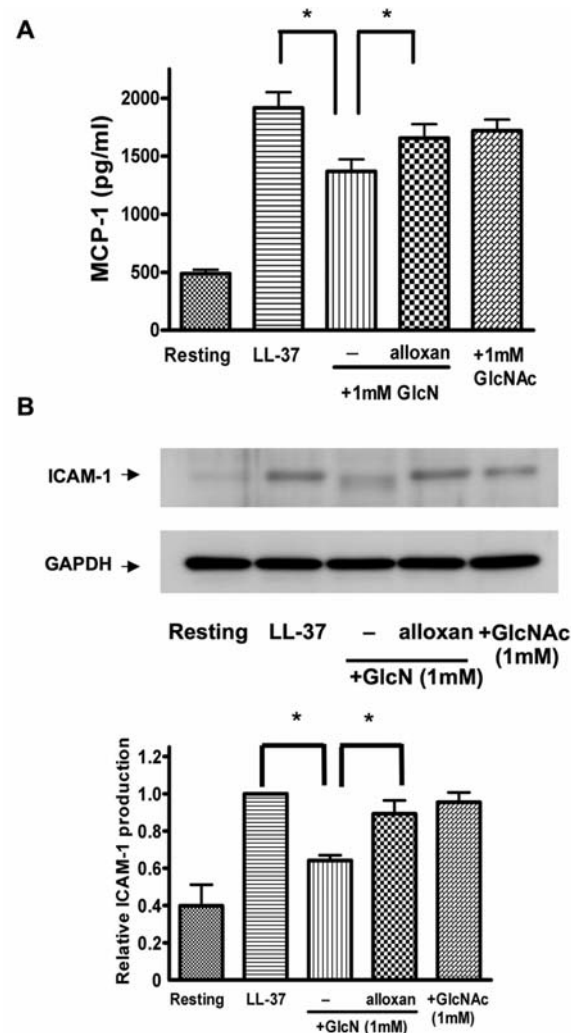
A

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Figure 4. Effect of alloxan on the glucosamine-induced suppression of MCP-1 and ICAM-1 expression. HUVEC were incubated without (Resting) or with 4 μM LL-37 (LL-37) in the absence or presence of 1 mM glucosamine (GlcN) or N-acetylglucosamine (GlcNAc). Alternatively, HUVEC were stimulated with 4 μM LL-37 in the presence of glucosamine and alloxan (0.5 mM). The supernatants were recovered, and MCP-1 was quantitated by ELISA (A). Cell lystaes were recovered, and ICAM-1 levels were evaluated by Western blotting (B). ICAM-1 expression was expressed as a ratio relative to LL-37-stimulation alone. Data are mean ± SEM from 6 independent experiments for both MCP-1 and ICAM-1; *p<0.05.

induced *O*-GlcNAc modification. Thus, we investigated the relationship between the glucosamine-induced *O*-GlcNAc modification and suppression of MCP-1 and ICAM-1 expression by using alloxan. Of importance, alloxan abrogated the glucosamine-induced suppression of MCP-1 and ICAM-1 expression (p<0.05) (Fig. 4) as well as glucosamine-induced *O*-GlcNAc modification (Fig. 3). These findings suggest the possible involvement of glucosamine-induced *O*-GlcNAc modification in the suppression of MCP-1 and ICAM-1 expression.

Discussion

LL-37 is the only cathelicidin member in humans, and exhibits antimicrobial activities against a broad spectrum of microbes in innate host defense system (7). Furthermore, LL-37 possesses the ability to chemoattract neutrophils,

monocytes and T lymphocytes (11). LL-37 is expressed in not only neutrophils and epithelial tissues but also infiltrating macrophages and endothelial cells in atherosclerotic lesions, suggesting that LL-37 plays a role in the inflammatory process of atherosclerosis (13,14). Edfeldt *et al* determined the effect of LL-37 on the atherosclerosis-related genes in HUVEC, and revealed that LL-37 can activate human endothelial cells and induce ICAM-1 and MCP-1 (13). As an adhesion molecule and a chemoattractant protein, they play important roles in the process of atherosclerotic plaque formation. Upon inflammatory activation, endothelial cells upregulate adhesion molecules (such as ICAM-1), and monocytes can bind to the endothelium through the adhesion molecules. Then, monocytes migrate into the arterial intima, and this process requires a chemoattractant (such as MCP-1) gradient. Once monocytes reside in the intima, they become intimal macrophages and internalize modified lipoproteins to be foam cells. Eventually, these macrophages congregate in a central core of atherosclerotic plaques (21).

Glucosamine is used to treat osteoarthritis (15), and it also exhibits anti-inflammatory actions. In brief, glucosamine suppresses neutrophil functions such as superoxide generation, granule enzyme release and chemotaxis (18). It also inhibits the aggregation of platelet (22). Glucosamine suppresses the cytokine-induced activation (such as nitric oxide, prostaglandin E_2, IL-8 production) of synoviocytes (23). Furthermore, glucosamine inhibits ICAM-1 expression in human retinal pigment epithelial cells (19).

In this study, we investigated the effect of glucosamine on the activation of endothelial cells, and revealed that glucosamine but not N-acetylglucosamine can inhibit the LL-37-induced expression of ICAM-1 and MCP-1 at both mRNA and protein levels (Figs. 1 and 2). In separate experiments, we stimulated endothelial cells with pro-inflammatory cytokines such as TNF-α. We found that TNF-α induces the expression of MCP-1 and ICAM-1, and the expression was similarly suppressed by glucosamine as observed in the LL-37-induced expression of MCP-1 and ICAM-1 (data not shown).

Various cytoplasmic and nuclear proteins are modified at serine and/or threonine residues by *O*-linked-N-acetylglucosamine monosaccharides (24). Mammalian cells contain *O*-GlcNAc transferase (OGT), an O-GlcNAc-modification forming enzyme and *O*-N-acetylglucosaminidase (*O*-GlcNAcase), an O-GlcNAc-modification degrading enzyme, and a gene disruption study shows that *O*-GlcNAc modification modulates cell functions (25). About one-quarter of identified *O*-GlcNAcylated proteins are involved in transcription or translation (20). Of note, it has been reported in the isolated heart that glucosamine increases cardiac *O*-GlcNAc levels and protects hearts from ischemia/reperfusion-injury, and that alloxan, an OGT inhibitor blocks the protection (26). Furthermore, in isolated cardiomyocytes, both glucosamine and O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino-N-phenylcarbamate (PUGNAc), an O-GlcNAcase inhibitor, show protective effect against hypoxia and reoxygenation stress (27). In addition, glucosamine improves cardiac function *in vivo* in a rat trauma/hemorrhage model, and this effect is associated with the increased cardiac *O*-GlcNAc levels (28). These observations

suggest that glucosamine is likely to circumvent the excessive cell activation under various diseased conditions via *O*-GlcNAc modification. Thus, we focused on the *O*-GlcNAc modification to estimate the mechanism for the glucosamine-induced suppression of endothelial cell activation.

The data demonstrated that glucosamine can induce the *O*-GlcNAc modification in HUVEC, but N-acetyl glucosamine can not (Fig. 3). Furthermore, alloxan, an OGT inhibiotor eliminated the inhibitory effect of glucosamine on ICAM-1 and MCP-1 expression (Fig. 4). Thus, because the effects of glucosamine, N-acetylglucosamine and alloxan on *O*-GlcNAc modification levels are negatively parallel with those on the endothelial cell activation, we hypothesized that *O*-GlcNAc modification is one of the possible mechanism for the glucosamine-induced suppression of endothelial cell activation. In conclusion, glucosamine can inhibit LL-37-induced expression of ICAM-1 and MCP-1, possibly via the *O*-GlcNAc modification. Usually, 1.5 or 3 g/day of glucosamine is administered to humans for treatment of osteoarthritis (18). We observed that serum glucosamine levels reached >0.02 mM after oral administration of 1.5 g glucosamine, as measured by a high performance liquid chromatography method using phenylisothiocyanate-derivatized glucosamine (29). Noticeably, in this study, glucosamine partially inhibited ICAM-1 and MCP-1 mRNA expression *in vitro* at concentrations of >0.01 mM. Thus, it is tempting to speculate that glucosamine affects endothelial cell activation *in vivo*, thereby possibly exhibiting anti-inflammatory action on atherosclerosis. In this context, it is interesting to note that glucosamine administration significantly reduces the atherosclerotic lesion in aortic root of apoE-deficient mice (30). The *in vivo* effect of glucosamine on atherosclerotic disorders should be carefully evaluated in the future.

Acknowledgements

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