ANTI-INFLAMMATORY AND ANTI-PLATELET ACTIONS OF GLUCOSAMINE

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Abstract

Glucosamine, a naturally occurring amino monosaccharide, is present in the connective and cartilage tissues, and contributes to maintaining the strength, flexibility and elasticity of these tissues. Thus, glucosamine has been widely used to treat osteoarthritis in humans. According to the recent biochemical and pharmacological findings, glucosamine normalizes cartilage metabolism, so as to inhibit the degradation and stimulate the synthesis of proteoglycans, and to restore the articular function.

In addition to the chondroprotective action, we have revealed that glucosamine can suppress neutrophil functions *in vitro* such as superoxide generation, phagocytosis, granule enzyme release and chemotaxis, suggesting that glucosamine could exhibit anti-inflammatory actions. Thus, we evaluated the effects of glucosamine on a DSS (dextran sulfate sodium)-induced rat colitis model. Interestingly, administration of glucosamine ameliorated the clinical symptoms and suppressed the pathological changes of colon in DSS-induced colitis. Moreover, glucosamine abrogated the activation of neutrophils (up-regulation of an adhesion molecule CD11b) *in vivo*.

Meanwhile, we have revealed that glucosamine can suppress platelet activation *in vitro* such as aggregation, release of granule contents and thromboxane A_2 production, suggesting that glucosamine could exert anti-platelet actions. Thus, we administered glucosamine to guinea pigs and examined its effects on platelet functions *ex vivo*. Concordant with the *in vitro* actions, glucosamine-administration suppressed platelet aggregation in response to ADP. Furthermore, glucosamine-administration inhibited the ADP-induced extracellular release of ATP and production of thromboxane A_2.

Altogether, these observations are likely to indicate that glucosamine could be expected as a therapeutic agent for treatment of inflammatory disorders and also a novel anti-platelet agent for treatment of thrombotic disorders, in addition to a chondroprotective agent.

Introduction

Chitin, a polymer of N-acetyl-glucosamine is present in the shells of shrimps and crab, and chitosan, a polymer of glucosamine is produced from the chitin by deacetylation under alkaline condition. In contrast, glucosamine-monomer is produced as glucosamine hydrochloride by hydrolysis and deacetylation of chitin under acidic condition. In our body, glucosamine is converted from glucose, and further metabolized to N-acetyl-glucosamine or N-acetyl-galactosamine, and functions as a component of glycosaminoglycans. Glycosaminoglycans, such as hyaluronic acid and chondroitin sulfate are present in the connective and cartilage tissues and contribute to maintaining the strength, flexibility, and elasticity of these tissues. Thus, glucosamine-supplement is widely used to treat osteoarthritis in humans as a precursor of glycosaminoglycans [1-3].

Several clinical trials in osteoarthritis have shown the significant symptom-relieving effects of glucosamine. Furthermore, recent biochemical and pharmacological studies have revealed that glucosamine normalizes cartilage metabolism, so as to stimulate the synthesis and inhibit the degradation of proteoglycans, and to restore the articular functions [4, 5]. In addition to the chondroprotective action, glucosamine is expected to exert anti-inflammatory actions. Thus, we previously revealed *in vitro* using human peripheral blood neutrophils that glucosamine can suppress neutrophil functions such as adhesion, chemotaxis, phagocytosis, granule enzyme release and superoxide production, and thereby possibly exhibits anti-inflammatory actions [6] (Fig. 1). Here, to demonstrate the anti-inflammatory actions of glucosamine *in vivo*, we first at the effects of glucosamine on an experimental model for inflammatory bowel disease, in which neutrophils are responsible for the tissue damage [7].

Furthermore, we previously found that glucosamine can suppress the aggregation of human platelets *in vitro*, suggesting that glucosamine could be a novel anti-platelet agent [8]. However, the effect of *in vivo* administration of glucosamine has not yet been determined. So, we orally administered glucosamine to animals, and examined its effects on platelet activation [9].

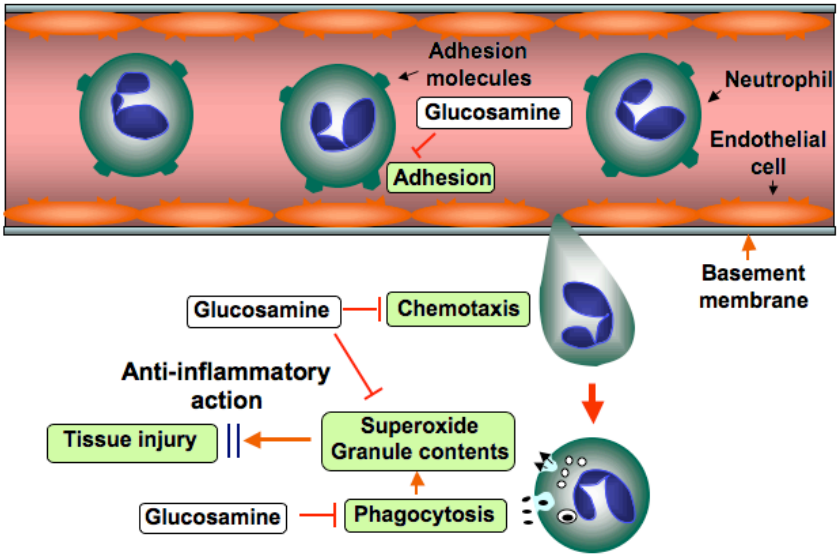

Figure 1 : Inhibitory actions of glucosamine on neutrophil functions.

Material and Methods

Dextran sulfate sodium (DSS)-induced colonic inflammation

Colitis was induced in Sprague-Dawley rats (300 g) by oral administration of 4% DSS solution for 7 days and 10 days intermittently, and clinical symptoms were evaluated, based on the disease activity index [10]. Glucosamine (400 mg/day) was orally administered everyday, and its effects on the disease activity index were evaluated. The index was calculated as the combined scores of weight loss, stool consistency and bleeding [10]. After the test period, the entire colons were excised, and the weight and length were measured. Furthermore, the activation of peripheral blood neutrophils was evaluated by flow cytometry, based on the expression of an adhesion molecule CD11b [6].

IL-8 production from colonic HT-29 epithelial cells

A human colonic epithelial cellline HT-29 was cultured in McCoy's 5A medium supplemented with 10% heat-inactivated FBS, penicillin (100 IU/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). In the presence of various concentrations of glucosamine, HT-29 cells were stimulated with LPS (100 ng/ml) or IL-1β (10 ng/ml), and the IL-8 levels in the culture supernatants were measured by ELISA. Furthermore, phosphorylation of ERK-1/-2 and p38MAPK was determined by western blotting using anti-phosphorylated ERK mAb and anti-phosphorylated p38MAPK mAB, respectively [6].

In vivo administration of glucosamine to guinea pigs

Glucosamine (400 mg/day) was orally administered to Hartley guinea pigs (250 g) for 22 days [9]. Thereafter, the platelet activation was determined *ex vivo*, based on the platelet aggregation, which was stimulated by ADP (2 μM), and monitored with an aggregometer. Furthermore, extracellularly released ATP was measured with luciferase, and thromboxane A_2 production was determined by measuring a stable metabolite thromboxane B_2 with ELISA [8, 9].

Quantification of plasma glucosamine

Glucosamine (400 mg) was administered to each animal by gastric gavage, and 30 min later blood was collected by cardiac puncture. Plasma glucosamine was quantitated by a high performance liquid chromatography method using phenylisothiocyanate-derivatized glucosamine [11].

Results and Discussion

Effect of glucosamine on DSS-induced colonic inflammation

First, we evaluated the effect of glucosamine on body weight-change in DSS-induced colitis. Body weight of naive control rats gradually increased during the test period. In contrast, the body weight change was suppressed in the colitis. Unexpectedly, the body weight change was not restored even by the administration of glucosamine.

Further, we evaluated the effect of glucosamine on the clinical symptoms of DSS-induced colitis by employing the disease activity index. In colitis rats, the index increased progressively, and reached 7 points on day 28. Importantly, glucosamine-administration (400 mg/kg) substantially suppressed the increase of index, and relieved the clinical symptoms of the colitis (Fig. 2).

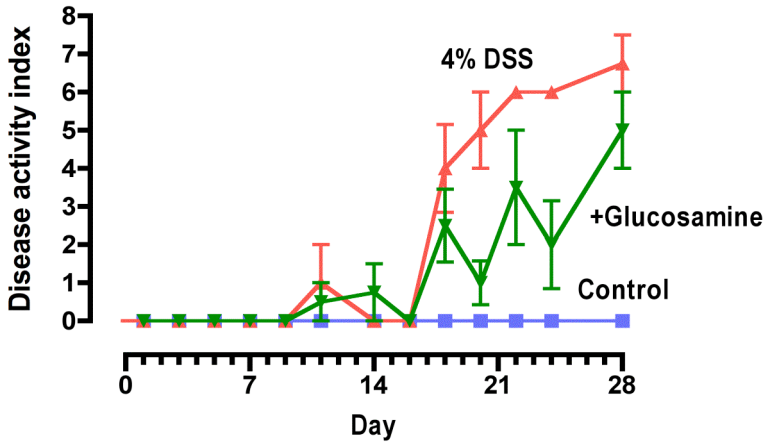

Figure 2 : Effect of glucosamine on disease activity index in DSS-induced colitis.

Moreover, we determined the effect of glucosamine on the colon weight/length in the colitis. In DSS-induced colitis, the weight of colon was increased, and the length was shortened by the inflammation. Thus, the index was enhanced. Interestingly, glucosamine-administration suppressed the inflammation, and restored this parameter.

Finally, we assessed the expression of CD11b as a marker of neutrophil activation. In colitis, neutrophils were activated, and the expression of CD11b was up-regulated. As expected, glucosamine-administration significantly suppressed the activation of neutrophils *in vivo*.

Furthermore, we determined the direct effect of glucosmaine on colonic epithelial cells using HT-29 cells. Both IL-1β and LPS induced the production of IL-8 from HT-29 cells. Interestingly, however, glucosamine dose-dependently suppressed the IL-8 production from these cells (Fig. 3).

Next, we examined the effect of glucosamine on the activation of signaling molecules. IL-1β- and LPS-stimulation induced the phosphorylation of ERK-1/-2 and p38MAPK in HT-29 cells.

Importantly, glucosamine dose-dependently suppressed the phosphorylation of ERK-1/-2 and p38MAPK.

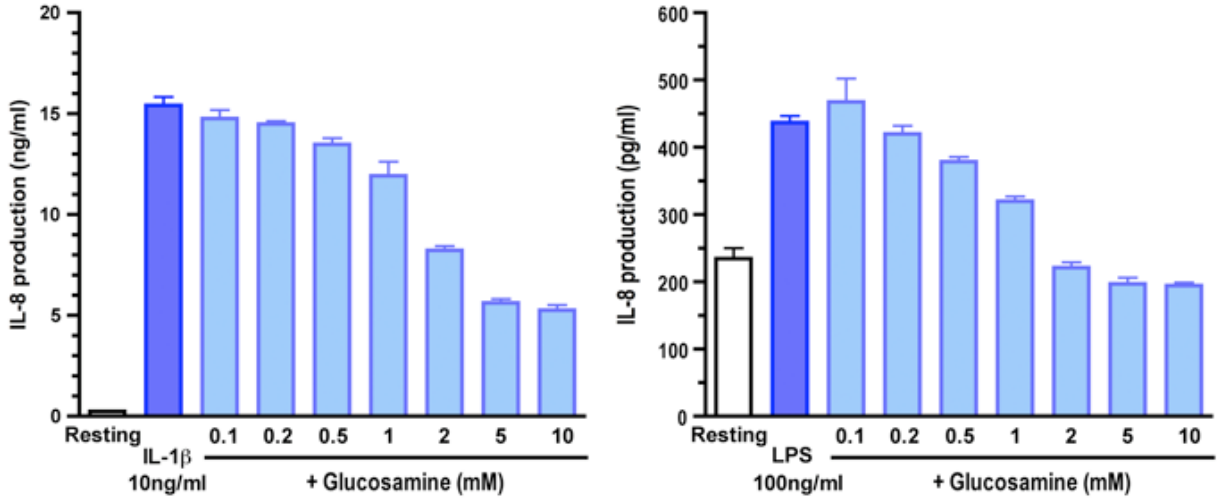

Figure 3 : Effect of glucosamine on IL-8 production from colonic HT-29 epithelial cells.

In summary, these observations indicate that glucosamine exhibits the protective actions on a colonic inflammation model, by restoring clinical symptoms and suppressing neutrophil activation. Furthermore, glucosamine can inhibit the production of IL-8 from colonic HT-29 epithelial cells, accompanied with the suppresed phosphorylation of MAP kinases. Thus, glucosamine is expected to exert the anti-inflammatory effects on inflammatory bowel diseases through the actions on neutrophils and epithelial cells.

Effects of glucosamine on platelet aggregation

We previously found that glucosamine can suppress the aggregation of human platelets *in vitro*, suggesting that glucosamine could be a novel anti-platelet agent [8]. However, the effect of *in vivo* administration of glucosamine has not yet been determined. So, glucosamine was orally administered to guinea pigs, and its effects on platelet activation were determined.

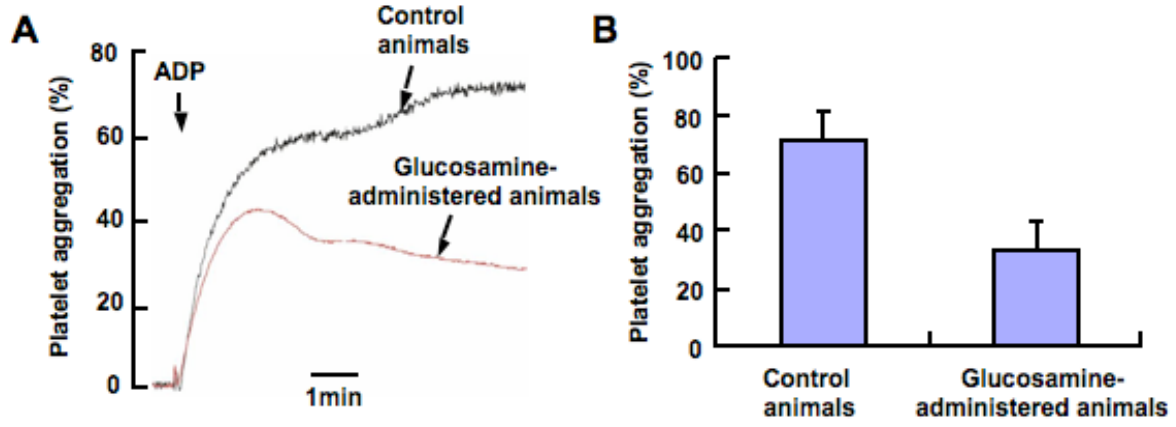

Figure 4 : Effect of oral administration of glucosamine on platelet aggregation.

As shown in Fig. 4, ADP induced the aggregation of platelets from control animals. Interestingly, however, the oral administration of glucosamine reduced the ADP-induced platelet aggregation about 50%. To further investigate the effect of glucosamine-administration on platelet aggregation, we utilized a different agonist, collagen. In contrast to the ADP-stimulation, collagen-induced platelet aggregation was not affected by the oral administration of glucosamine.

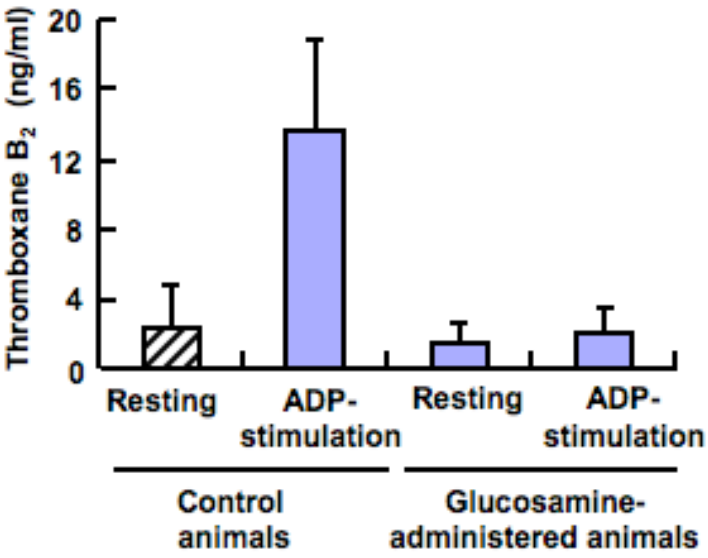

Figure 5 : Effect of oral administration of glucosamine on thromboxane production by platelets.

Next, to evaluate the effect of glucosamine-administration on thromboxane A_2 production, we measured the levels of thromboxane B_2, a stable metabolite of thromboxane A_2 in the supernatants of platelets. Platelets from control animals produced approximately 13 ng/ml of thromboxane B_2 in response to ADP. However, platelets from glucosamine-administered animals did not produce thromboxane B_2 upon stimulation (Fig. 5).

Furthermore, to examine the effect of glucosamine-administration on the extracellular release, we measured the amounts of ATP in the supernatants of platelets. In response to ADP, platelets from control animals released approximately 0.8 μM ATP. However, platelets from glucosamine-administered animals did not essentially release ATP upon stimulation.

Next, to estimate the plasma level of glucosamine, 400 mg glucosamine was administered to each animal by gastric gavage, and its plasma levels were measured by HPLC. As a result, the glucosamine level reached approximately 1.4 mM after the administration. In contrast, glucosamine was not detected in the plasma of control animals. Thus, we measured the platelet aggregation *in vitro* in the presence of various concentrations of glucosamine. Importantly, 0.1 mM and 1 mM glucosamine, namely the plasma levels of glucosamine, significantly inhibited the ADP-induced platelet aggregation.

ADP activates platelets via the binding of ADP to its receptors [12]. So, to clarify the inhibitory mechanism of glucosamine, we analyzed the ADP-binding to the receptors by using tritium-labeled ADP as a ligand. Scatchard analysis indicated that there are two kinds of receptors, high and low affinity binding sites for ADP on the platelets (Fig. 6). Notably, glucosamine affected the binding to the low affinity receptors without affecting the high affinity receptors. Based on these data, we calculated the dissociation constants (K_d) and binding sites for the two receptors. Importantly, glucosamine more intensely reduced the binding for the low affinity receptors than the high affinity receptors, without significant effect on the dissociation constants for ADP. This type of inhibition indicates a noncompetitive type of inhibition, in which the inhibitor binds with either the receptor or the agonist-receptor complex.

Furthermore, we evaluated the body-weights, platelet counts and bleeding time after the glucosamine-administration. As expected, there were no substantial differences between control and glucosamine-administered animals in these parameters. These data indicate that glucosamine has no serious adverse effect and is expected as a safe agent. Similarly, long-term clinical trials with oral administration of glucosamine for 3 years to humans indicated that no apparent side effects were recorded and routine laboratory tests did not show any abnormalities during treatment of osteoarthritis [3].

	High affinity		Low affinity	
	Kd (μM)	Binding sites (sites/cell)	Kd (μM)	Binding sites (sites/cell)
Control	0.127	8611	1.73	98933
+1mM GlcN	0.123	7520	1.40	63034

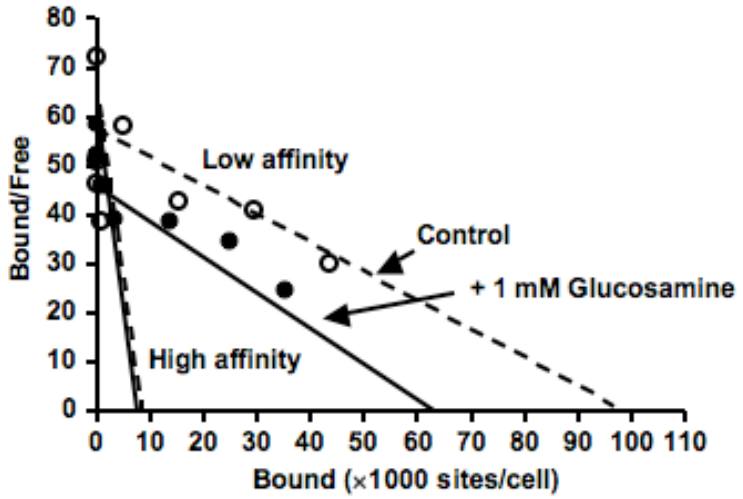

Figure 6 : Effect of glucosamine (GlcN) on the binding of [^{3}H]-ADP to platelets.

Finally, to determine the effect of *in vivo* administration of glucosamine to humans, we evaluated platelet aggregation using blood samples from human volunteers after oral administration of 1.5 g glucosamine per day for one week. Notably, ADP-induced platelet aggregation was decreased about 30% after oral administration of glucosamine, compared with that before the administration.

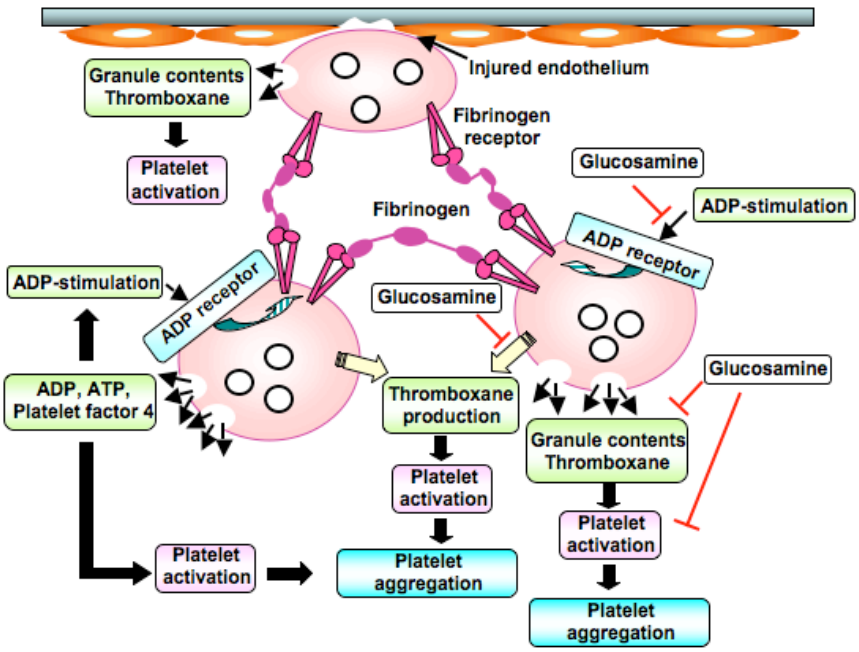

Figure 7 : Effects of glucosamine on glatelet functions. When activated, platelets extracellularlly release granule contents such as ATP, and produce thromboxane. These substances subsequently activate platelets to induce aggregation. Glucosamine possibly suppresses the ADP-binding to the receptors, and thereby inhibits the release of granule contents, thromboxane production and platelet aggregation.

In summary, these observations indicate that oral administration of glucosamine suppresses the ADP-induced platelet aggregation, extracellular release of granule contents (ATP) and thromboxane production *ex vivo*. Furthermore, binding study using tritium-labeled ADP revealed that glucosamine inhibits the binding of ADP to its receptors (Fig. 7). Thus, glucosamine is expected to moderately suppress platelet activation *in vivo*, possibly via the inhibition of ADP binding to its receptors.

Conclusions

The present studies indicate that in addition to a chondroprotective action, glucosamine could be expected to exert anti-inflammatory and anti-platelet actions *in vivo* as a therapeutic agent for inflammatory and thrombotic disorders.

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