Glucosamine, a naturally occurring amino monosaccharide, suppresses dextran sulfate sodium-induced colitis in rats

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Received March 21, 2008; Accepted May 19, 2008

DOI: 10.3892/ijmm_00000025

Abstract. Glucosamine, a naturally occurring amino monosaccharide, is widely used to treat osteoarthritis in humans. Furthermore, glucosamine exhibits an anti-inflammatory action by inhibiting the activation of neutrophils, chondrocytes and synoviocytes. Recently, we revealed that glucosamine suppresses cytokine-induced activation of intestinal epithelial cells *in vitro*. In the present study therefore, we investigated whether glucosamine exhibits the anti-inflammatory effect *in vivo*, using dextran sulfate sodium (DSS)-induced colitis in rats, a model of inflammatory bowel diseases (IBD). The results indicated that glucosamine improved the clinical symptoms (evaluated by disease activity index), and suppressed colonic inflammation (evaluated by colon length and weight/length ratio) and tissue injury (evaluated by histological damage score) in DSS-induced colitis. Furthermore, glucosamine inhibited the activation of intestinal epithelial cells, as evidenced by the suppressed phosphorylation of NF-κB in the intestinal mucosa of DSS-induced colitis. Collectively, these observations suggest that glucosamine is likely to suppress the activation of intestinal epithelial cells *in vivo*, thereby possibly exhibiting anti-inflammatory action in a DSS-induced rat colitis model. Thus, glucosamine could prove to be a useful agent for IBD.

Introduction

Inflammatory bowel diseases (IBD) are chronic recurrent disorders of the intestinal tract due to a dysregulated mucosal immune response with unknown etiology (1). They are characterized by a marked infiltration of neutrophils into mucosal lesions of the colon, accompanied by epithelial cell necrosis and ulceration (2), with neutrophils playing an important role in the pathogenesis of IBD (3). Currently, medical treatment of IBD relies mainly on traditional drugs: aminosalicylates, corticosteroids and immunosuppressants (4). These drugs reduce inflammatory injury and attenuate the expression of some proinflammatory molecules. However, their side effects severely disturb the quality of life of patients, particularly during long-term treatment (5). The development of an optimal therapy for IBD is therefore important.

Glucosamine, a naturally occurring amino monosaccharide, is present in 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 (6). Several short- and long-term clinical trials in osteoarthritis have shown the significant symptom-modifying effect of glucosamine (7-9). According to recent biochemical and pharmacological findings, the administration of glucosamine normalizes cartilage metabolism, so as to inhibit the degradation (10) and stimulate the synthesis of proteoglycans (11,12), and to restore articular function.

In addition to its chondroprotective action, glucosamine is supposed to exert an anti-inflammatory action by inhibiting neutrophil functions such as superoxide generation, phagocytosis, granule enzyme release and chemotaxis (13). Glucosamine has also been demonstrated to suppress interleukin-1ß (IL-1ß)-induced cyclooxygenase-2 (COX-2) expression in chondrocytes and synoviocytes (14), and exhibit preventive actions on rheumatoid arthritis (15) as well as adjuvant arthritis, in rats (16). We recently demonstrated the suppressive effect of glucosamine on intestinal epithelial cell activation; glucosamine suppresses tumor necrosis factor-α (TNF-α)-induced production of interleukin-8 (IL-8), expression of intercellular adhesion molecule-1 (ICAM-1), phosphorylation of p38 MAPK (mitogen-activated protein kinase) and NF-κB (nuclear factor-κB) in intestinal epithelial HT-29 cells (17). Thus, glucosamine could potentially exert an anti-inflammatory action on IBD by inhibiting intestinal epithelial cell activation.

In DSS-induced colitis, DSS is orally administered to rodents (rats and mice), which exhibit severe colorectal

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Key words: glucosamine, inflammatory bowel diseases, dextran sulfate sodium-induced colitis, intestinal epithelial cells

damage mimicking IBD in humans (1,18,19). In the present study, we therefore investigated the anti-inflammatory effect of glucosamine *in vivo*, using DSS-induced colitis in rats.

Materials and methods

Reagents. Dextran sulfate sodium salt (molecular weight 36-50 kDa; reagent grade) was purchased from MP Biomedicals LLC (Solon, OH). D-Glucosamine hydrochloride was supplied by Koyo Chemical Co., Ltd., (Tokyo, Japan).

Animals and induction of colitis. Specific pathogen-free male Sprague-Dawley (SD) rats weighing 280-310 g were purchased from Charles River Laboratories Japan, Inc., (Yokohama, Japan). The animals were housed under pathogen-free conditions (controlled temperature of 23±1˚C and humidity of 50±10%), and fed standard laboratory food and water *ad libitum*. In accordance with institutional guidelines (Juntendo University, School of Medicine), the animals received proper care and maintenance and the experiments were performed based on the guidelines of the International Association for the Study of Pain (20). Rats (n=23) were randomized into three groups: the DSS group administered with only DSS (n=8), the glucosamine group with glucosamine and DSS (n=8), and control groups with distilled water (n=7). To elicit colitis, rats were administered with 4% DSS *ad libitum* for 7 days, followed by administration with only distilled water for 7 days, and further administered with DSS for 11 days. Rats were also administered with glucosamine (1%, 10 mg/ml) dissolved in 4% DSS, or distilled water *ad libitum* for the experimental period. The intake volume of glucosamine solution was measured on alternate days, and animals received an average of 40 ml glucosamine solution (400 mg glucosamine per animal per day). Changes in body weight and development of clinical symptoms were evaluated every other day during the experimental period.

Clinical analysis. Colitis was evaluated using a disease activity index (DAI), as described by Cooper *et al* (21) with a slight modification, using parameters of body-weight loss, stool consistency and bleeding. Briefly, no weight loss was scored as 0 points; weight loss of 1-5%, 1 point; loss of 6-10%, 2 points; 11-20% weight loss, 3 points; and a loss of >20% of the weight was scored as 4 points. Stool was characterized as normal (0 points), loose (2 points), or diarrhea (4 points). No bleeding was scored 0 points, and gross bleeding was scored 4 points. The score was added to obtain DAI ranging from 0 (healthy) to 12 (maximal activity of colitis). Rats were sacrificed by diethyl ether inhalation after the experimental period, and then the colon was quickly removed, opened longitudinally, and gently cleared of stool for morphologic studies. The length and weight of the colon were measured, and tissue obtained from each colon was processed for further assays.

Histological assessment of colitis. Colon tissues were fixed in 20% buffered formalin and finally embedded in paraffin. After deparaffinizing of tissue sections (3-μm) on glass slides, the sections were stained with hematoxylin and eosin. Microscopic sections were examined, and histological scoring was performed, as described by Ohkawara *et al* (22). In brief, tissue damage was categorized into six grades: 0, normal mucosa; 1, infiltration of inflammatory cells; 2, shortening of the crypt by less than half; 3, shortening of the crypt by more than half; 4, crypt loss; 5, destruction of epithelial cells.

Immunohistochemical detection. Colon tissue sections (3 μm) on glass slides were deparaffinized and rehydrated. Then, they were autoclaved for 10 min in 0.01 M citrate buffer (pH 6.0) and cooled for 10-20 min at room temperature. After washing with PBS, the sections were incubated with rabbit polyclonal anti-phosphorylated NF-κB p65 antibody (1:50, Ser276; Cell Signaling Technology, Danvers, MA) for 35 min at room temperature in an automated staining system (Ventana *i*view DAB Universal Kit, Ventana Medical systems, Tucson, AZ) according to the manufacturer's recommendations. Thereafter, the slides were washed three times with PBS and incubated with goat anti-rabbit biotinylated antibody (Vector Laboratories, Burlingame, CA). Tissue sections were visualized by incubating with diaminobenzidine tetrahydrochloride containing 0.03% hydrogen peroxide for 20 min, and counterstained with hematoxylin.

Flow cytometric analysis of CD11b expression. Blood samples were collected from tail vein under anesthesia. The heparin-anticoagulated blood cells (100 μl) were added with 2 μg phycoerythrin (PE)-labeled mouse anti-rat CD11b/c monoclonal antibody (Caltag Laboratories, Burlingame, CA) on ice for 30 min. Erythrocytes were removed by mixing with lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$ and 100 μM EDTA) for 15 min on ice (23). After washing twice in PBS-1% BSA, cells were analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA) using Cell Quest software (Becton Dickinson). CD11b/c expression was presented as mean fluorescence intensity (MIF).

Measurement of serum cytokines. Serum cytokines were quantified by a sandwich enzyme-linked immunosorbent assay (ELISA) using a Ready-SET-Go! ELISA set for TNF-α (eBioscience, San Diego, CA) and DuoSet ELISA Development kits for IL-6 and cytokine-induced neutrophil chemoattractant factor 1 (CINC-1, CXC chemokine family) (R&D Systems, Minneapolis, MN). The detection limits of these cytokines were <16 pg/ml.

Statistical analysis. The data are expressed as the mean ± SEM. Statistical analyses were performed using the unpaired Student's t-test or one-way ANOVA (and nonparametric). A p-value <0.05 was considered to be statistically significant.

Results

Effect of glucosamine on DAI of DSS-induced colitis in rats. Recently, we reported that glucosamine suppresses intestinal epithelial cell activation by inhibiting the production of chemokines, expression of adhesion molecule, and activation of signaling molecules (17). In this study, to determine whether glucosamine has a protective action *in vivo*, glucosamine was orally administered to rats with DSS-induced colitis, an IBD model (24). Accordingly, we first evaluated the effect of glucosamine on DSS-induced colitis using DAI as a

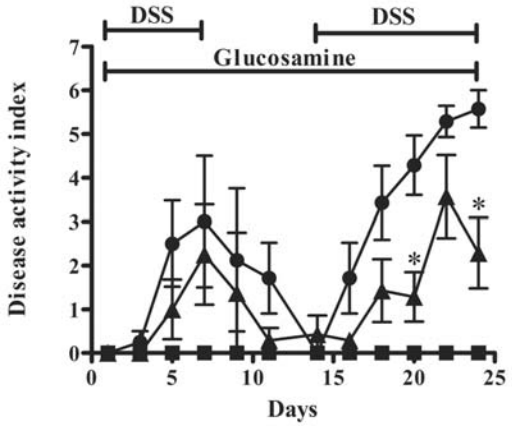

Figure 1. Effect of glucosamine administration on disease activity index (DAI) in DSS-induced colitis. Colitis was induced by administering DSS for 7 days, followed by distilled water for 7 days and further DSS for 11 days *ad libitum*. Glucosamine (1%, 10 mg/ml) dissolved in 4% DSS, or distilled water was also administered *ad libitum* for the experimental period. Colitis was evaluated using DAI with parameters of body-weight loss, stool consistency and bleeding. Data represent the means ± SEM of 7-8 animals in the control (square), DSS (circle) and glucosamine groups (triangle). Values are compared between DSS and glucosamine groups. $^{*}p<0.05$.

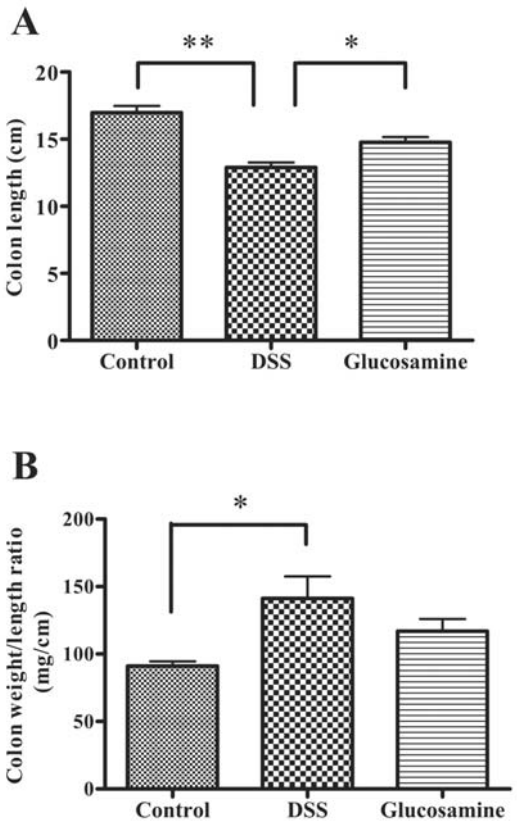

Figure 2. Effect of glucosamine administration on the pathological changes in DSS-induced colitis. The colon was removed from rats, opened longitudinally, and gently cleared of stool for morphologic studies. The length (cm) of the colon (A), and the weight/length ratio of the colon (cm/mg) (B) were measured. Data represent the means ± SEM of 6-7 animals in an experimental group. Values are compared among the control, DSS and glucosamine groups. $^{*}p<0.05$, $^{**}p<0.001$.

parameter, which provides a scoring system to quantify disease severity that correlates with histopathological damage of intestinal mucosa (1,25). Colitis was induced by giving DSS for 7 days, followed by giving distilled water for 7 days and further giving DSS for 11 days. As shown in Fig. 1, DAI began to increase from day 3 and reached a first peak on day 7. After the termination of the first administration of DSS (day 7), clinical symptoms slowly improved: DAI declined and reached the basal level on day 14. When colitis was induced again by the second administration of DSS, DAI further increased, compared with that for the first administration of DSS. Of note, glucosamine administration suppressed the DSS-induced increase of DAI during the experimental period (days 20 and 24, $p<0.05$).

Effect of glucosamine on histopathological changes in DSS-induced colitis. DSS administration induces shortening of the colon and increase in weight/length ratio (mg/cm) (26). Thus, we evaluated the effect of glucosamine on these parameters for colitis. Colon was significantly shortened by DSS-treatment (Fig. 2A, $p<0.001$). Notably, glucosamine administration reversed the DSS-induced shortening of colon (Fig. 2A, $p<0.05$). Furthermore, the colonic weight/length ratio was increased in the DSS-administered group (Fig. 2B, $p<0.05$). Interestingly, glucosamine administration moderately suppressed the increase in weight/length ratio (Fig. 2B, DSS group, 141.3±16.4 vs. glucosamine group, 116.9±9.1 mg/cm).

Furthermore, the damage of intestinal mucosa was microscopically evaluated. In the DSS group, several erosions and ulcers, and crypt destruction were observed in the colon (Fig. 3C and D). In the glucosamine group, ulceration was markedly suppressed; however, erosions were sometimes observed, and the submucosal layer was still edematous (Fig. 3E and F), compared with the control group (Fig. 3A and B). In addition, the severity of tissue damage was evaluated by using histological scoring of hematoxylin- and eosin-stained sections. Histological damage score of the colitis was dramatically increased in the DSS group compared with that of the control group (Fig. 4, $p<0.001$). Consistent with the clinical features, histological damage score was significantly decreased in rats treated with glucosamine, compared with that of the DSS group (Fig. 4, $p<0.05$).

Effect of glucosamine on the phosphorylation of NF-κB in DSS-induced colitis. NF-κB has been reported to be activated in inflamed colonic mucosa of IBD (27,28). To determine the effects of glucosamine on the phosphorylation of NF-κB in DSS-induced colitis, colon sections were immunohistochemically evaluated. As shown in Fig. 5, phosphorylation of NF-κB was enhanced in the epithelial cells of DSS-induced colitis, compared with that of the control group. Of note, glucosamine administration apparently suppressed the phosphorylation of NF-κB. These observations suggest that glucosamine possibly inhibits the DSS-induced activation of signaling molecules (such as NF-κB) in intestinal epithelial cells.

Effect of glucosamine on CD11b expression on neutrophils in DSS-induced colitis. We previously reported that glucosamine inhibits the activation of neutrophils *in vitro* (13). We therefore investigated the effect of glucosamine administration on

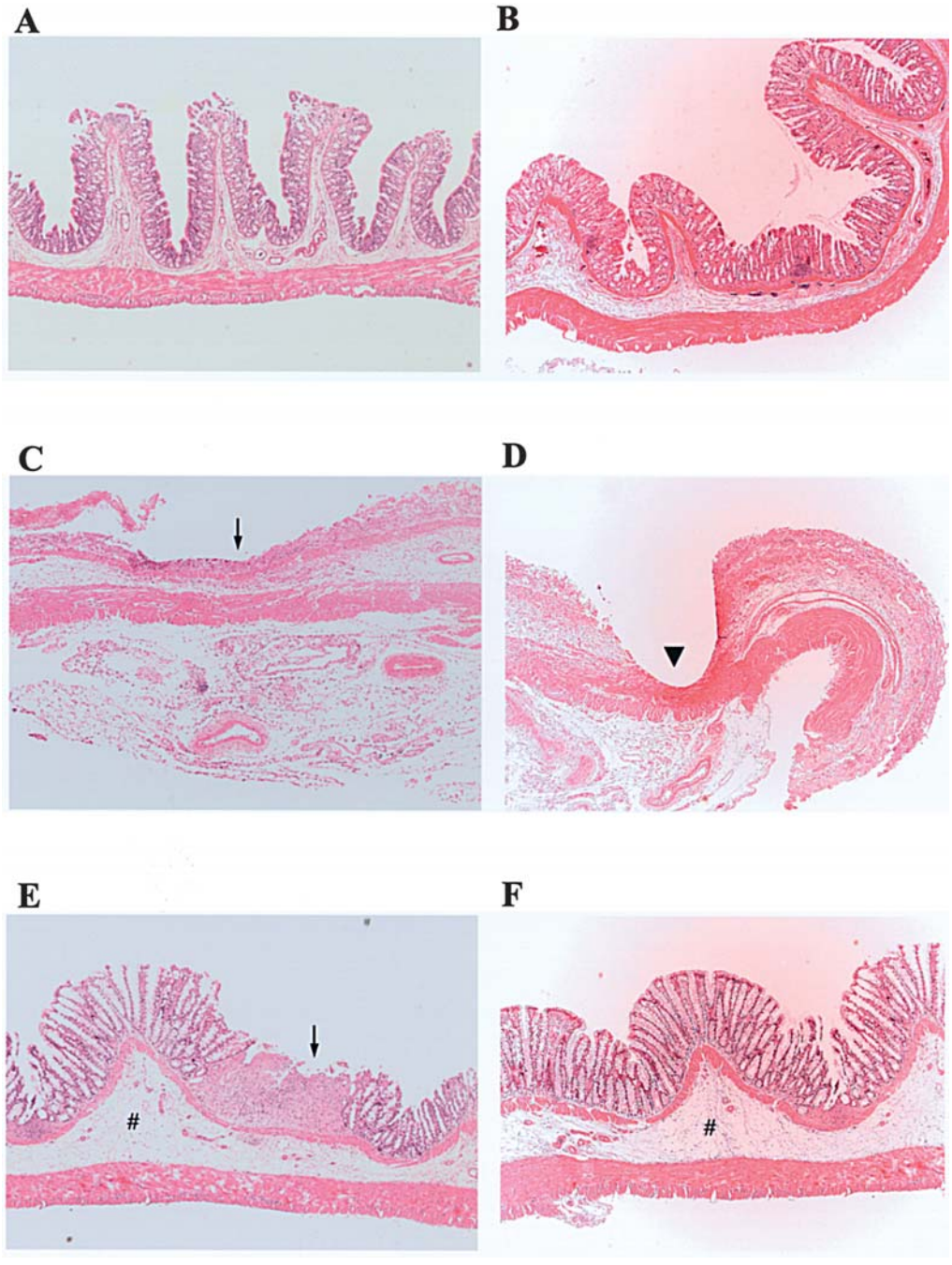

Figure 3. Effect of glucosamine administration on histopathological changes in DSS-induced colitis. The colon was fixed, and the tissue sections (3 μm) were stained with hematoxylin and eosin. Erosion and ulcer are indicated by arrows and an arrowhead, respectively. The symbol (#) indicates edematous submucosal layer. Data are from one of 3-4 rats in the control (A and B), DSS (C and D) and glucosamine groups (E and F). Original magnification x40.

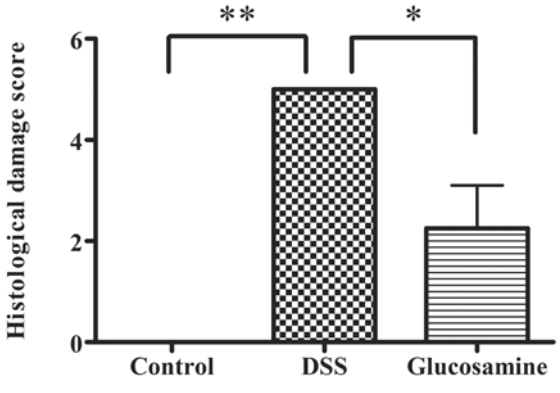

Figure 4. Effect of glucosamine administration on histological damage score of intestinal mucosa in DSS-induced colitis. Hematoxylin- and eosin-stained sections were microscopically evaluated by histological damage score. Data represent the means ± SEM of 3-4 animals in the control, DSS and glucosamine groups. Values are compared among control, DSS and glucosamine groups. $^{*}p<0.05$, $^{**}p<0.001$

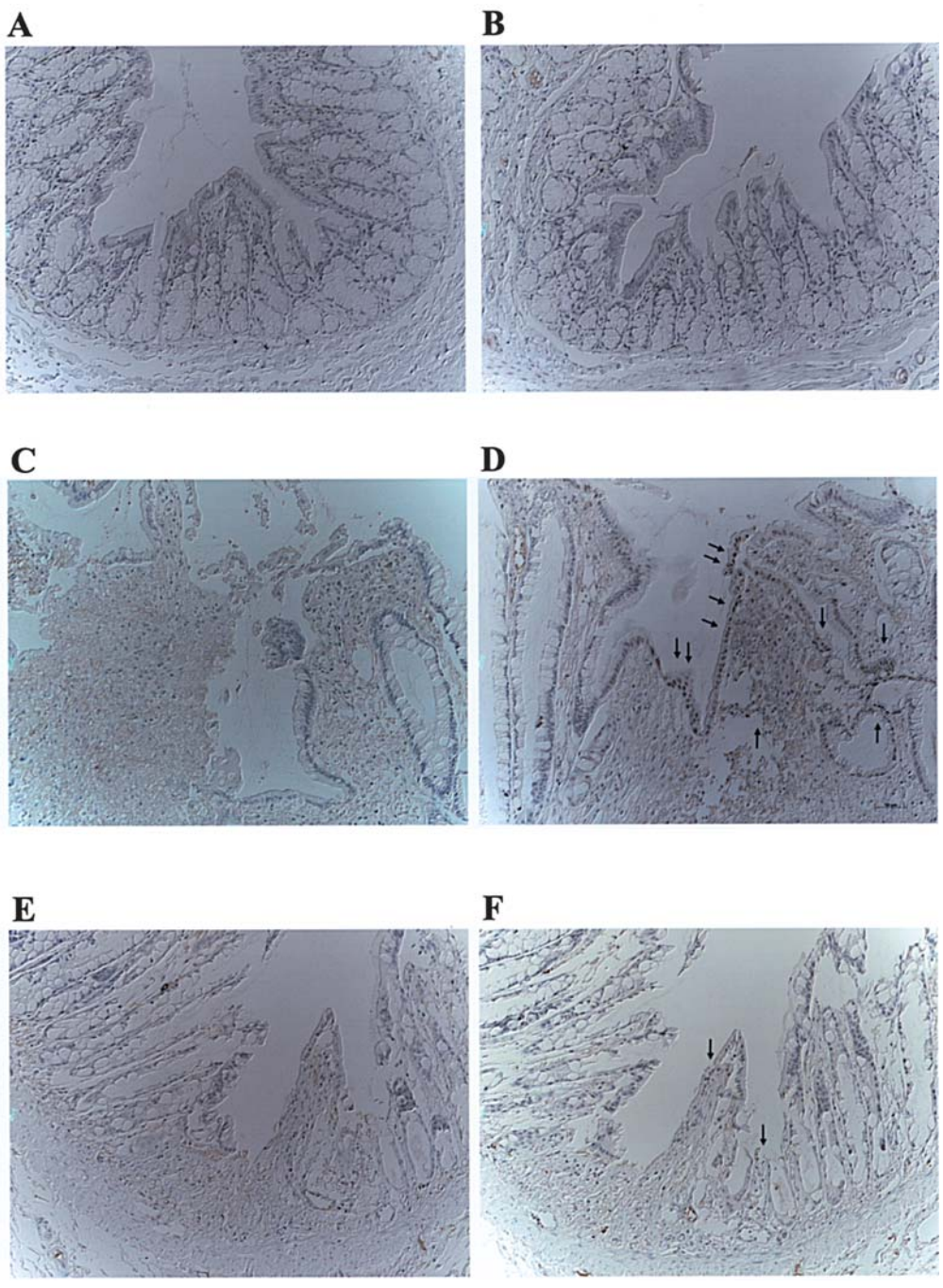

Figure 5. Effect of glucosamine administration on the phosphorylation of NF-κB in DSS-induced colitis. Phosphorylated NF-κB p65 was immunohistochemically detected by using rabbit anti-phosphorylated NF-κB p65 antibody (B, D and F) in colon sections. Tissue sections were also incubated with normal rabbit IgG (A, C and E). Epithelial cells with phosphorylated NF-κB p65 are indicated by arrows. Data are from one of 3-4 rats in control (A and B), DSS (C and D) and glucosamine groups (E and F). Original magnification x100.

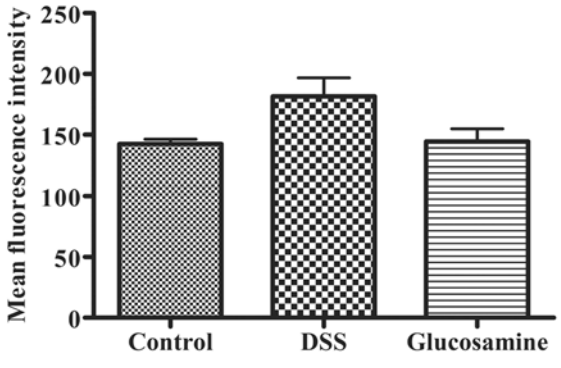

Figure 6. Effect of glucosamine administration on CD11b/c expression on neutrophils in DSS-induced colitis. Heparin-anticoagulated blood cells (100 μl) were added with PE-labeled mouse anti-rat CD11b/c monoclonal antibody, and CD11b/c expression was analyzed by flow cytometry after hemolysis of erythrocytes. The cells were gated to identify neutrophil population, and 10,000 cells were analyzed in each sample. CD11b/c expression was presented as the mean fluorescence intensity. Data represent the means ± SEM of 3-4 animals in control, DSS and glucosamine groups.

neutrophil activation in DSS-induced colitis using CD11b/c as a marker. As shown in Fig. 6, the expression of CD11b/c was slightly increased in the DSS group (181.6±15.3 MIF), compared with that in the control group (142.4±3.9 MIF). Of interest, glucosamine administration substantially suppressed DSS-induced increase of CD11b/c expression (144.4±10.4 MIF).

These observations suggest that glucosamine is also likely to modulate neutrophil activation in a DSS-induced colitis model.

Effect of glucosamine on proinflammatory cytokines level in DSS-induced colitis. Proinflammatory cytokines and chemokines are important molecules in the pathogenesis of IBD (29,30). Thus, we determined the levels of TNF-α, IL-6 and CINC-1 by ELISA using sera obtained from DSS-induced colitis rats on day 25. Unexpectedly, TNF-α and IL-6 were not detected; their levels were below the detection limit. In contrast, CINC-1 was detected, and the level was increased in DSS-induced colitis rats (122.9±35.7 pg/ml, n=7), compared with that in the control rats (103.0±7.8 pg/ml, n=7). Significantly, CINC-1 level was reduced by glucosamine administration (67.9±12.8 pg/ml, n=6).

Discussion

IBD (such as ulcerative colitis and Crohn's disease) are chronic recurrent disorders of the intestinal tract. To investigate their pathogenesis, several experimental models have been developed (24). Among these is DSS-induced colitis, in which DSS is orally administered to rats and mice which exhibit severe colorectal damage mimicking IBD in humans (1,18,19). Therapeutically beneficial substances for human IBD are shown to reduce disease activities in DSS-induced models (1,24,31).

Glucosamine, a naturally occurring amino monosaccharide, is widely used to treat osteoarthritis in humans. Furthermore, glucosamine exhibits an anti-inflammatory action by inhibiting neutrophil functions (13) and suppressing interleukin-1ß (IL-1ß)-induced cyclooxygenase-2 (COX-2) expression in chondrocytes and synoviocytes (14). Furthermore, we revealed that glucosamine suppresses cytokine-induced activation of intestinal epithelial cells *in vitro* (17). Thus, in the present study, we investigated whether glucosamine administration has an anti-inflammatory effect on IBD, using DSS-induced colitis in rats. The results indicated that glucosamine clinically improved symptoms evaluated by DAI, pathologically inhibited colonic inflammation evaluated by colon length and weight/length ratio, and microscopically reduced tissue injury evaluated by histological damage score in DSS-induced colitis. Furthermore, glucosamine inhibited the activation of intestinal epithelial cells, as evidenced by the suppression of DSS-induced phosphorylation of NF-kB in the intestinal mucosa. Moreover, glucosamine administration substantially inhibited the activation of neutrophils, as evidenced by the suppression of DSS-induced increase of CD11b/c expression on peripheral blood neutrophils. IBD are characterized by a marked infiltration of activated neutrophils into colon mucosal lesions, where epithelial cells are activated to produce various inflammatory mediators (18,27,30). These findings suggest that glucosamine is likely to suppress the activation of intestinal epithelial cells and neutrophils *in vivo*, thereby possibly exhibiting an anti-inflammatory action in a DSS-induced colitis model.

Proinflammatory mediators including cytokines and chemokines are implicated in the pathogenesis of IBD (3,27,29-32). We therefore determined the levels of proinflammatory cytokines (TNF-α and IL-6) and chemokine (CINC-1) by ELISA in sera from DSS-induced colitis rats. TNF-α and IL-6 were not detected; however, CINC-1 was detected, and was increased in DSS-induced colitis rats. Significantly, CINC-1 level was reduced by glucosamine administration. CINC-1 is a member of the CXC chemokine family and is thought to play a critical role in initiating neutrophil recruitment in inflammation site (27,33). Thus, our observations suggest that glucosamine may also exert an anti-inflammatory action in a DSS-induced colitis model by suppressing inflammatory mediator production.

Glucosamine has been widely used to treat osteoarthritis in humans (6). Moreover, glucosamine has been demonstrated to exhibit preventive actions on rheumatoid arthritis in humans (15). The present study provides novel evidence that glucosamine attenuates the inflammatory responses in a DSS-induced colitis model by suppressing the activation of epithelial cells (as evidenced by the phosphorylation of NF-κB) and possibly neutrophils (as evidenced by the expression of CD11b/c). Thus, glucosamine could prove to be a useful agent for IBD. The effect of glucosamine on human IBD should be carefully evaluated in the future.

Acknowledgements

This work was supported in part by a grant from the Foundation for Pre-symptomatic Medicine and Anti-aging, Institute of Gerontology, Hakujikai, Japan.

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