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Glucosamine sulfate promotes osteoblastic differentiation of MG-63 cells via anti-inflammatory effect

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Abstract—Glucosamine sulfate (SGlc) has been known to be effective in controlling osteoarthritis (OA) symptoms in several clinical studies. However, the mechanisms of this positive effect of SGlc in human OA still remain elusive. Therefore, first, the effects of SGlc on the differentiation of osteoblast-like MG-63 cells were investigated. Our results demonstrate that SGlc can increase ALP activity, collagen synthesis, osteocalcin secretion, and mineralization in osteoblastic cells in vitro. Furthermore, it was observed that SGlc exhibited anti-inflammatory effect on production of TNF-α, IL-1β, and PGE_2 in macrophage, RAW264.7 cells. In conclusion, these results suggest that SGlc can promote cell differentiation in cultured MG-63 osteoblast cells via anti-inflammatory effect.

Osteoarthritis (OA) is one of the most prevalent chronic inflammatory diseases and its incidence increases with age. Its most prominent feature is the progressive destruction and loss of articular cartilage which results in impaired joint motion, severe pain, and, ultimately, disability that pose a significant public health problem.[1] The degeneration of matrix components of articular cartilage is accompanied with an excess production of pro-inflammatory cytokines.[2] Interleukin-1 beta is widely accepted as one of the proinflammatory cytokines that plays a major role in the pathophysiology of OA.[3] It induces a cascade of catabolic events in chondrocytes including the up-regulation in genes of cyclooxygenase and the release of PGE_2.[4] Among these pharmacological treatments, nonsteroidal anti-inflammatory drugs (NSAIDs), despite serious adverse effects associated with their long-term use, remain among the most widely prescribed drugs for OA.[5] Thus, new research efforts have been made to find out strategies to prevent the development and progression of OA. Many clinical trials have tested this hypothesis and Glc supplements are widely used to relieve arthritic complaints.[6] To meet the demand for Glc nutritional supplements, three forms of Glc are commonly available: Glc hydrochloride, SGlc, and *N*-acetyl-glucosamine. These Glc compounds are generally derived from chitin, a biopolymer present in the exoskeleton of marine invertebrate animals. Recently, SGlc was found to be effective in controlling OA symptoms in several clinical studies.[7] SGlc is a sugar, a sulfated aminomonosaccharide, one of the constituents of the disaccharide units present in articular cartilage proteoglycans. In vitro study has shown that it can alter chondrocyte metabolism, and this is the rationale usually given for its use in OA.[8] SGlc has been reported to be effective in cell culture and animal model system and was widely used to treat the symptoms of OA as a nutritional supplement.[9] However, the detailed mechanisms of the beneficial effect of SGlc in human OA joint are still obscure. In view of the clinical evidence that SGlc may benefit patients with OA, it is worthwhile to consider the mechanism for its effectiveness. In particular, studies focusing on osteoblast and macrophage are lacking. Therefore, in the present study, we examined osteoblast differentiation and anti-inflammatory effects of SGlc using biological assays.[10]

In order to prepare SGlc, Glc was sulfated by SO_3-pryidine complex according to the method of Ronghua et al.[11] The structure of purified SGlc was determined by proton (^1H)/carbon (^{13}C) NMR spectroscopy (JNM-ECP-400 (400 MHz) spectrometer, JEOL, Japan), elemental (C, N, and H) analysis (Elementar Analysesysteme, Elementar Vario, EL, USA), and IR spectroscopy (Spectrum 2000 FT-IR spectrophotometer,

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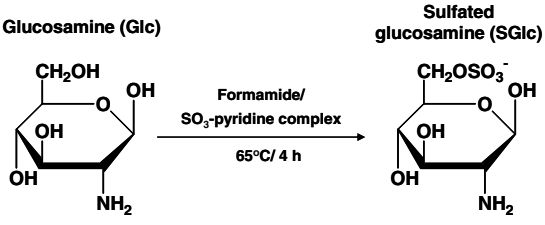
Perkin-Elmer, USA). FT-IR (KBr, r, cm^{-1}): 3434 (s, O–H), 2985 and 2755 (w, C–H), 1686 (m, C–O), 1480 (m, C–H), 1255 (s, S–O), 1064, 1010 (s, pyranose), 815 (s, S–O) (14); ^{13}C NMR (400 MHz, D_2O, y, ppm): 52.1 (C-2), 67 (C-6), 69, 76 and 72 (C-3, 4, 5), 89, 92 (C-1) (15); elemental analysis: S% (11.13), C% (24.98), N% (5.04), H% (4.4). SGlc was dissolved and then diluted with the medium. All other reagents were from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated (Scheme 1).

First, in order to investigate the effects of SGlc on the differentiation of osteoblast-like cells, cell growth, ALP activity, collagen synthesis, and osteocalcin secretion were evaluated in human osteoblastic MG-63 cells. Our data in this study demonstrate that SGlc can increase ALP activity, collagen synthesis, and osteocalcin secretion in osteoblastic cells in vitro. These results support that the SGlc could prevent the progression of OA through improvement of the function of osteoblastic cells at low concentrations. Previous study reported that sulfated polysaccharides enhance the osteoblast differentiation through increase of bone morphogenetic proteins.[12]

MG-63 cells were incubated with SGlc and cell viability was measured by MTT assay. As shown in Figure 1, SGlc did not exert any cytotoxic effect on MG-63 cells even at the highest tested concentration (1000 μg/mL) like Glc. Therefore, based on this preliminary viability data, concentrations of SGlc ranging from 1 to 1000 μg/mL were selected to investigate the effect on osteoblastic differentiation and inflammation.

First of all, ALP activity was measured to study the effect of SGlc on the osteoblastic differentiation in MG-63 cells. As shown in Figure 2a, the intact cells in the presence of Glc and SGlc (5–100 μg/mL) caused a significant increase in the ALP activity. SGlc exerted a concentration-dependent effect on ALP activity and increased significantly it ($P < 0.01$), compared with Glc. However, as shown in Figure 2b, there was no effect on ALP activity in lysate of cells treated with Glc even at 100 μg/mL. On the other hand, the effect of SGlc in increasing ALP activity was observed at 5 μg/mL of concentration. SGlc at higher concentration (1000 μg/mL) does not have a promotive effect on cell growth. However, it was shown that SGlc at lower concentration (5 μg/mL) clearly increased ALP activity in both intact osteoblastic cells and cell lysate. Our finding in present study suggests that the difference in ALP activity of cell lysate between Glc and SGlc treatment groups was attributed to the occupancy of the $–OSO_3^-$ group that offers the unique opportunity to have a substantial affinity to ALP, thereby activates enzyme. Therefore, a beneficial effect of SGlc in OA may be based partly on activation of ALP. Our finding suggests that SGlc might play an important role in promoting osteoblastic differentiation of osteoblastic cells.



Scheme 1. Synthesis of glucosamine sulfate from glucosamine hydrochloride.

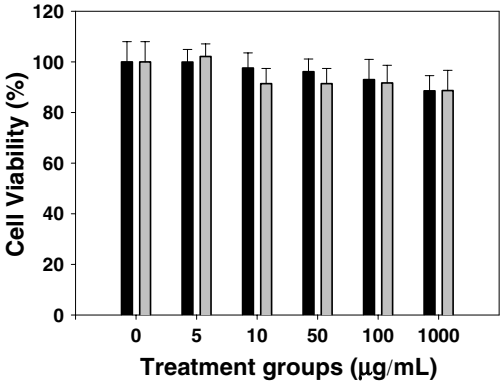


Figure 1. Effects of SGlc on viability of MG-63 cells in the presence of FBS. Cells were treated with SGlc and cell viability was determined by MTT assay after 24 h. Glc was used as a positive control. Data represent means ± SE of at least three independent experiments calculated as a percentage of blank.

The MG-63 cells were treated with various concentrations of SGlc (10–1000 μg/mL) and the content of osteocalcin in medium was measured as shown in Figure 3. There was no significant difference between Glc and SGlc treatment group. Both Glc and SGlc exhibited a concentration-dependent effect on osteocalcin secretion in MG-63 cells. However, the effects of SGlc in greatly elevating collagen synthesis as well as osteocalcin secretion in osteoblastic cells were not observed in Glc treatment group. This finding in this study is consistent with several investigations indicating that SGlc has mild to large beneficial effects in OA.[13–15]

The effect of SGlc on collagen synthesis in osteoblastic MG-63 cells is shown in Figure 4. The collagen synthesis of cells was significantly increased by the addition of SGlc ($P < 0.01$), compared with Glc. Glc did not exhibit a significant increase in collagen synthesis below 100 μg/mL of SGlc. Collagen synthesis was significantly increased with concentration increment of SGlc. Addition of SGlc at 10 μg/mL enhanced collagen synthesis by about three times in comparison with Glc. In order to examine the effect of SGlc on mineralization in MG-63 cells, mineralization was detected with Alizarin red staining. MG-63 cells were cultured for 7 days in the presence of SGlc and β–glycerophosphate. As shown in Figure 5, both Glc and SGlc groups showed clearer red color than blank group. The mineralization level of cells treated with SGlc was significantly increased ($P < 0.01$), compared with Glc. The results of mineralization experiments clearly indicated that SGlc could induce osteoblast differentiation. Mineralization as well as increase of ALP activity were features indicating a positive role of SGlc in osteoblast differentiation. In our study, SGlc led to a marked increase in mineralization in MG-63 cells. These results

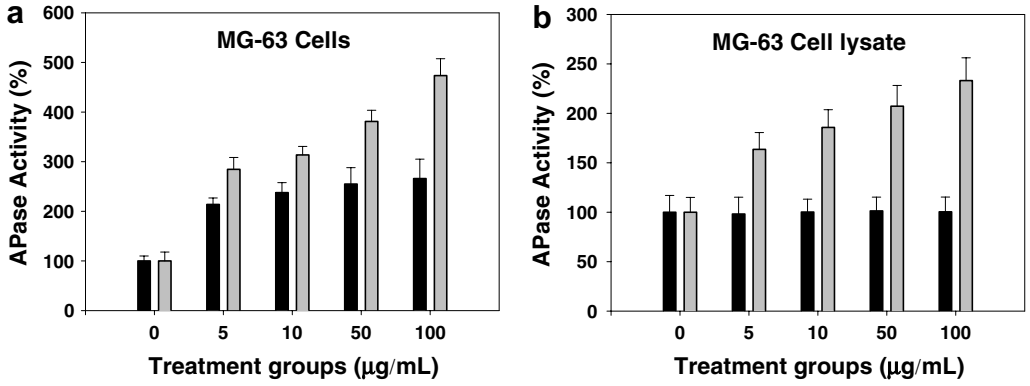


Figure 2. Effects of SGlc on the alkaline phosphatase activity in MG-63 cells. Cells were treated with SGlc at 5, 10, 50, 100, and 500 μg/mL in the presence of FBS for 24 h. Alakaline phosphatase activity was determined in both intact cells (a) and cell lysate (b). Glc was used as a positive control. Data represent means ± SE of at least three independent experiments calculated as a percentage of blank.

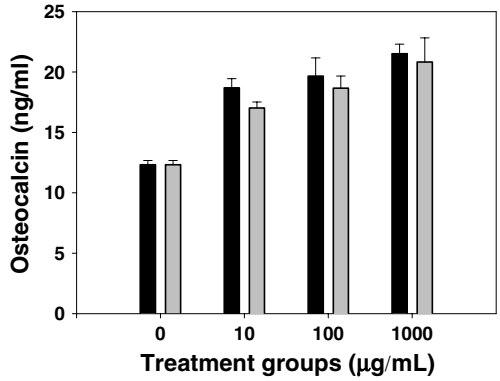


Figure 3. Effects of SGlc on the osteocalcin secretion of MG-63 cells. Cells were treated with SGlc at 5, 10, 50, 100, and 500 μg/mL in the presence of FBS for 4 days. Glc was used as a positive control. Data represent means ± SE of at least three independent experiments calculated as a percentage of blank.

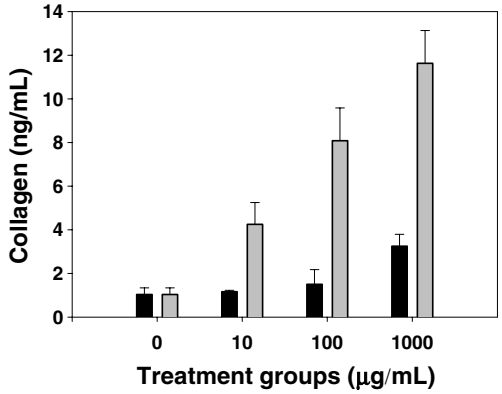


Figure 4. Effects of SGlc on the collagen synthesis of MG-63 cells. Cells were treated with SGlc at 5, 10, 50, 100, and 500 μg/mL in the presence of FBS for 4 days. Glc was used as a positive control. Data represent means ± SE of at least three independent experiments calculated as a percentage of blank.

confirmed that SGlc might have a therapeutic potential in patients with OA by stimulating production of proteoglycan.[16]

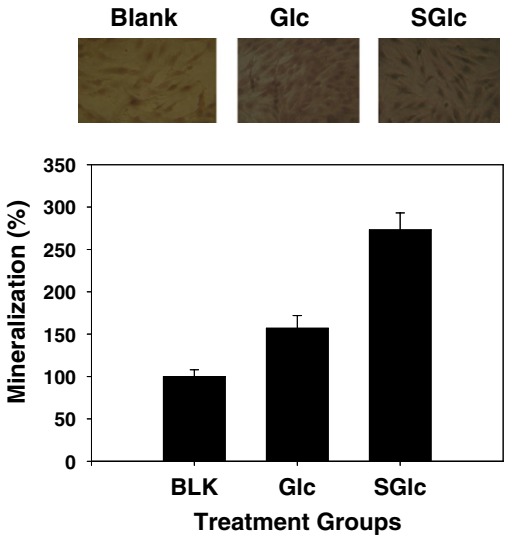


Figure 5. Alizarin red staining 7 days after cultures: photograph of representative stained cell culture. MG-63 cells were cultured for 7 days in the presence of SGlc, fixed, and stained to detect mineralization using Alizarin red as described in Materials and methods. Three plates represent blank, Glc, and SGlc treatment group, respectively. Positive results are shown as red area. Same experiments have been performed for three times.

However, the mechanisms through which SGlc may exert these effects are not still clarified. Recently, it is known that some bone-resorbing agents like TNF-α act on osteoblasts and stimulate PGE_2 release from osteoblasts.[17] Furthermore, the presence of PGE_2 was reported to cause a significant decrease in bone ALP activity and a corresponding increase in bone acid phosphatase activity. The inhibitory effects of SGlc on production of inflammatory mediators such as TNF-α, IL-1β, and PGE_2 following treatment of lipopolysaccharide at 1 μg/mL were investigated in RAW 264.7 cells, as shown in Figure 6. First of all, as shown in Figure 6a, pretreatment of Glc and SGlc significantly inhibited TNF-α synthesis stimulated by lipopolysaccharide ($P < 0.01$) compared with blank group. Moreover, it was observed clearly that the inhibitory effect of SGlc

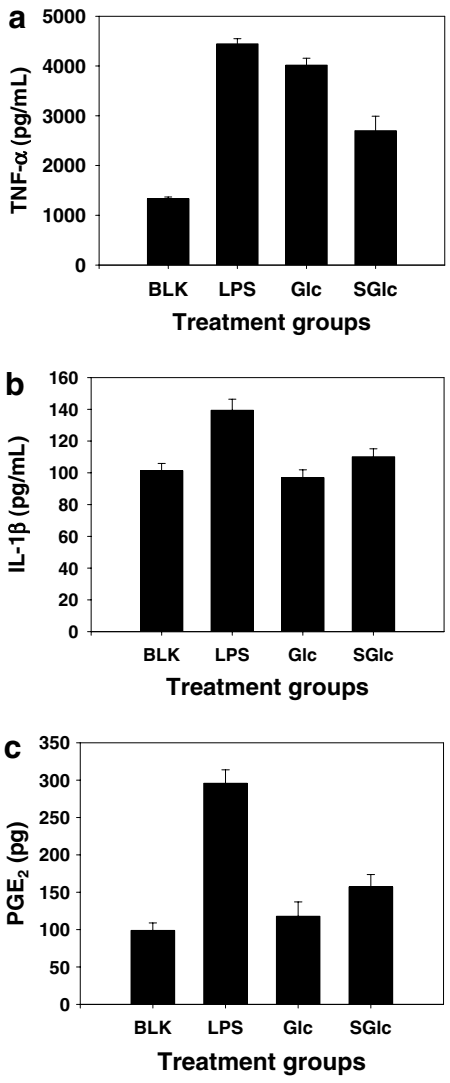


Figure 6. Anti-inflammatory effects of SGlc in RAW 264.7 cells. Cells were pretreated with SGlc at 5, 10, 50, 100, and 500 μg/mL for 1 h before stimulation with lipopolysaccharide. Inflammatory mediators such as TNF-α, IL-1β, and PGE_2 were determined by immunoassay. Glc was used as a positive control. Data represent means ± SE of at least three independent experiments calculated as a percentage of blank.

on TNF-α synthesis was greatly increased by 4 times compared with Glc treatment group. As shown in Figure 6b, IL-1β synthesis was also significantly reduced following treatment of Glc and SGlc. However, there was no significant difference in IL-1β synthesis stimulated by lipopolysaccharide between Glc and SGlc treatment groups. PGE_2 is an inflammatory mediator to be involved in osteoclast function. As shown in Figure 6c, addition of Glc and SGlc at 10 μg/mL reduced PGE_2 production in RAW 264.7 cells stimulated by lipopolysaccharide. However, there was no significant difference in PGE_2 production between Glc and SGlc treatment groups. PGE_2level by treatment of SGlc was decreased by 100%, compared with blank group. In the present study, SGlc clearly inhibited TNF-α production induced by lipopolysaccharide in macrophage. The result suggests that SGlc may have an important role in the regulation of localized bone destruction associated with inflammatory diseases, such as rheumatoid arthritis, by inhibition of TNF-α production in macrophages. In addition to TNF-α, IL-1β has been reported to play a critical role in the inflammatory process and the connective tissue destruction observed in OA.[18] Our data showed that SGlc can inhibit IL-1β production by lipopolysaccharide in macrophages. This result is related to previous report that IL-1β is a proinflammatory cytokine released by synovial cells, chondrocytes, and invading macrophages in inflamed joints.[19,20] Previous study has shown that IL-1β retards anabolic activities of the chondrocytes leading to declines in proteoglycan synthesis and collagen synthesis.[21,22] However, the mechanisms associated with these ways of action for SGlc still remain elusive. In the present work, we have demonstrated that SGlc inhibits PGE_2 synthesis induced by lipopolysaccharide in human osteoblast-like cells. This result reveals that the inhibitory effect of SGlc on PGE_2 production in osteoblastic cells may lead to stimulation of mineralization as reported in previous study about effect of PGE_2 on mineralization.[23]

To date, most of the clinical studies examining the effect of Glc on OA have been performed with either the sulfate or the chloride salts of Glc. The mechanisms by which Glc influences osteoarthritis are not currently known. Glc, as a constituent of glycosaminoglycans, can increase proteoglycan synthesis.[24] Recently, Glc has been shown to suppress the excess production of nitric oxide that is implicated in the pathogenesis of arthritis.[25] In addition, it was reported that Glc hydrochloride inhibits production of prostaglandin E_2 by interleukin-1 β and matrix metalloproteinases from normal chondrocytes and synoviocytes.[26,27] A limitation of in vitro studies showed that *N*-acetylglucosamine was more efficient than Glc in inhibiting interleukin-1 β-mediated activation of human articular chondrocytes in vitro.[28] In addition to these direct effects on synthesis and degradation of GAGs, NAG is capable of scavenging reactive oxygen species, such as superoxide anion, hydrogen peroxide, and hydroxyl radical.[29] There is significantly more evidence supporting SGlc than the other forms for OA. Therefore, we were interested in studying only SGlc that exits as the anion at physiologic pH because of its acidic sulfate groups, and is usually administered as the sodium salt. Sulfate is needed for the production of a component of articular cartilage such as proteoglycan. Some researchers now feel that the sulfate part of SGlc may be responsible for its effect on osteoarthritis because the anti-arthritis benefits of sulfur are so well documented that many arthritis patients find relief with methylsulfonylmethane, which is a concentrated source of sulfur.[30–32] Our results indicated that its sulfate groups greatly stimulate mineralization by osteoblasts compared with Glc, and reduce the catabolic processes in the osteoblast by inhibiting the activity of inflammatory mediators such as TNF-α, IL-1β, and PGE_2 released from macrophages. Therefore, it is suggested that the anti-inflammatory effect of SGlc can play a major role in inhibition of OA.

In conclusion, our findings provide the first experimental evidence that SGlc exerts a stimulatory effect on differentiation in osteoblast-like MG-63 cells in vitro via anti-inflammatory effect on macrophage. Therefore, we suggest that SGlc can be used as a therapeutic agent for the prevention and treatment of OA.

Acknowledgment

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10. Biological assays: MG-63, osteoblast-like cells, and RAW264.7 cells were suspended in medium and plated at a density of 10^3 cells/well into a 96-well plate. After 24 h, SGlc at different concentrations was treated. Cell proliferation was measured by MTT assay. Cell lysate and the collected medium from MG-63 cells were used for the measurement of ALP activity and protein concentration. Cellular collagen content was measured using Metra CICP EIA kit (Qudel Inc., USA) and osteocalcin content in culture medium was measured using osteocalcin immunoassay kit (Bioresource Inc., USA). Mineralization was detected with Alizarin red staining of calcium. The Alizarin red concentration was determined by measuring the absorbance at 562 nm. RAW264.7 cells were pretreated with SGlc at different concentrations for 1 h before lipopolysaccharide treatment. TNF-α, IL-1β, and PGE_2 contents in culture medium were measured using enzyme immunoassay kit (Amersham Biosciences, Sweden). Comparisons of all data were performed using two-tailed, unpaired Student's t test. A P value less than 0.05 was considered statistically significant. Data are expressed as means ± SE.
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