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Sulfated glucosamine inhibits MMP-2 and MMP-9 expressions in human fibrosarcoma cells

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Abstract—In the present study, sulfated glucosamine (SGlc) that has been reported to relieve joint pain and inflammation in many arthritis patients was studied for its inhibitory effects on MMP-2 and MMP-9 in human fibrosarcoma cells. Expression and activity of above MMPs studied using gelatin zymography suggested SGlc as a potent MMP inhibitor. Further, transfection of promoter genes of MMPs and their transcription factors clearly exhibited that inhibition of MMP-2 and MMP-9 was due to down-regulation of transcription factor, NF-κB. However expression of activator protein-1 (AP-1), another important transcription factor of MMPs, was not affected by SGlc treatment. In addition, protein expression results of Western blot analysis were also in agreement with the results of gene transfection experiments. Moreover, down-regulation of NF-κB resulted in production of low levels of both NF-κB p50 and p65 proteins and directly affected activation process of MMP-2 and MMP-9 expressions. Since MMPs involve in joint inflammation, it can be presumed that inhibition of MMP-2 and MMP-9 can be one of the mechanisms of SGlc to be an effective drug in relieving the symptoms of osteoarthritis.

1. Introduction

Matrix metalloproteinases (MMPs), also known as matrixins, are a family of zinc-containing endopeptidases that share common structural domains and have the capacity to degrade extracellular matrix (ECM) components, as well as alter their biological functions.[1] The MMPs are categorized simplistically into three major functional groups, in part based on substrate specificity. The interstitial collagenases (MMP-1, -8, and -13), that preferentially have affinities toward collagen types I, II, and III, the stromelysins (MMP-3, -10, and -11) with specificity for laminin, fibronectin, and proteoglycans, and the gelatinases (MMP-2 and -9), which most effectively cleave type IV and V collagen are the main three groups.[2] Regulation of gene expression of most MMPs is controlled by two major transcription factors, NF-κB and AP-1.[3] MMP promoter contains an AP-1 binding consensus site at −79 upstream from the starter site and further upstream there is a cluster of regulatory elements including another AP-1 binding site and NF-κB binding site. However depending on the type of MMP, these transcription factors affect differentially to regulate their gene expressions. Under normal physiological conditions, MMP transcripts are generally expressed at low levels, but these levels rise rapidly when tissues are locally induced to undergo remodeling events such as inflammation, wound healing, cancer, and arthritis.[4] MMPs including MMP-2 and MMP-9 contribute to joint destruction in rheumatoid arthritis (RA) by directly degrading the cartilage and gelatin matrix and indirectly promoting angiogenesis. Therefore inhibition of MMPs is a primary therapeutic target in RA and improvements in therapeutic benefit may be achieved by targeting specific MMPs. A subclass of MMPs, the gelatinases (MMP-2 and MMP-9), contribute directly to joint destruction as well as being vital during angiogenesis. Therefore, inhibition of MMP-2 and MMP-9 may represent a selective approach to improve therapeutic benefits in RA.

Sulfated glucosamine (SGlc) or glucosamine sulfate has been identified to be an effective slow-acting drug in relieving the symptoms of OA.[5] However, up-to-date studies have not been carried out to identify the inhibitory effects of SGlc on MMPs, that might be one of its

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potential influences to act as anti-OA agent. Therefore, for the first time here in this study we tried to identify the inhibitory effects of SGlc on two gelatinases, MMP-2 and MMP-9 in human fibrosarcoma (HT1080) cells.

2. Results and discussion

2.1. Sulfated glucosamine (SGlc) is non-toxic to fibroblasts

Synthesis of SGlc was performed under mild conditions as described in the experimental section and structural data confirmed successful introduction of sulfate group to the 6th carbon of Glc. At the initial experiments, different concentrations of SGlc were treated to HT1080 cells growing in serum containing or serum-free media and its effect on cell viability was determined. As depicted in Figure 1, none of the tested concentrations exerted any toxic effect under both serum-free and serum containing conditions. In addition to human fibrosarcoma (HT1080) cells, effects of SGlc on the viability of normal human fibroblasts (MRC-5) were also tested. Similarly, SGlc did not exert any toxic effect on MRC-5 cells (data not shown). Therefore, the concentrations used for MMPs expression experiments were non-toxic and the observed results were not due to any toxic influence.

2.2. SGlc is a potent inhibitor of MMP-2 and MMP-9 in HT1080 cells

Fibroblasts are traditionally defined as the cells that produce collagens and are considered to be the primary source of most extracellular matrix (ECM) components.[6] In addition, these cells secrete matrix degradative enzymes, MMPs, relatively in large amounts. As such, fibroblasts play a central role in tissue remodeling and wound healing processes mainly by producing MMP-2 and MMP-9, and are involved in the pathogenesis of connective tissue diseases. Among the different kinds of fibroblasts, human fibrosarcoma (HT1080) cells are commonly used to study MMPs. Therefore, in this study we used the same cell line to study the effects of SGlc on MMPs. Inhibition of MMPs can take place at different levels, from their gene expression to enzyme activity. Since MMP-2 and MMP-9 belong to the group of MMPs that use gelatin as their substrate, gelatin zymography was employed to screen their activity. For that, HT1080 cells were pre-treated with different concentrations of SGlc and MMPs expressions were stimulated by potent tumor promoter, phorbol 12-myristate 13-acetate (PMA). As shown in Figure 2, about 3-fold increment in MMP-9 expression was observed following stimulation of cells with PMA. In contrast, comparatively very low MMP-2 expression was observed and it was unable to quantify by densitometry. Low expression of MMP-2 compared to that of MMP-9 following activation with PMA is commonly observed in HT1080 cells and that may be due to differential

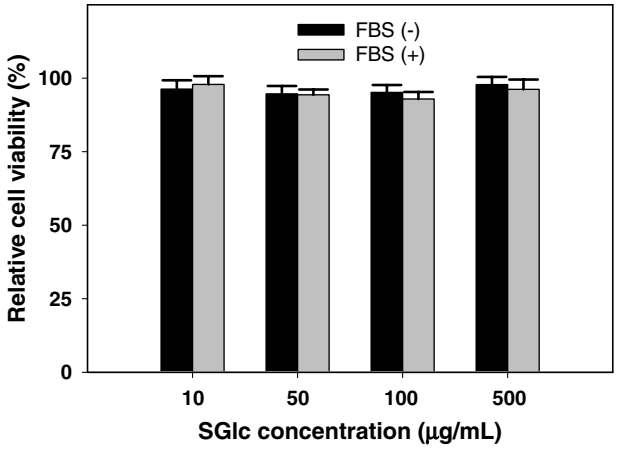

Figure 1. Effect of SGlc on the viability of HT1080 cells. Cells were treated with different concentrations of SGlc for 24 h under serum containing or serum-free conditions and cell viability was assessed by MTT assay as described in the experimental section. Results of independent experiments were averaged and represented as percentage cell viability compared to the viability of non-treated blank cells.

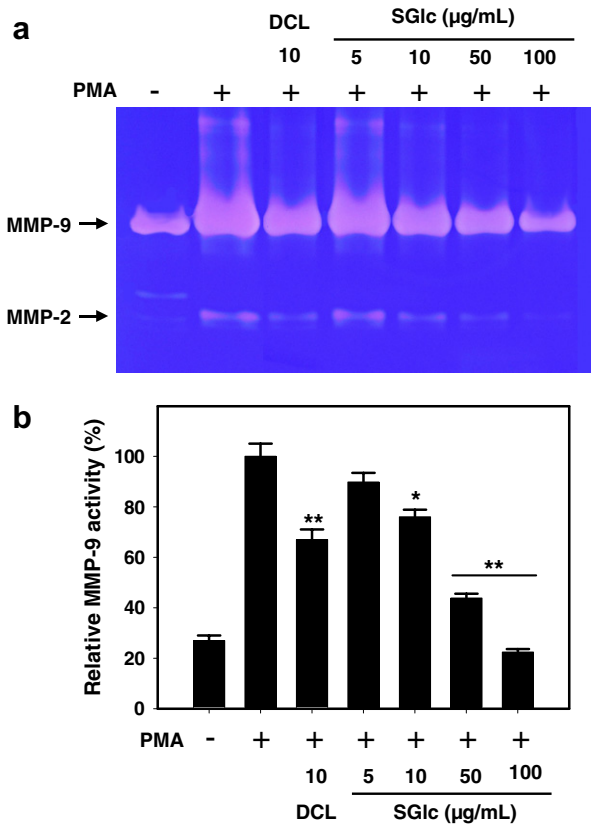

Figure 2. Gelatin zymography for the determination of MMP-2 and MMP-9 activities in SGlc treated HT1080 cells. (a) Gelatinolytic activities of MMP-2 and MMP-9 in conditioned media were detected by electrophoresis of soluble protein on a gelatin containing 10% polyacrylamide gel. (b) Areas and relative intensities of gelatin-digested bands by MMP-9 were quantified by densitometry and expressed as relative MMP-9 activity compared to that of PMA-alone treated cells. DCL was used as a positive control and the results of independent experiments were averaged and compared statistically ($^{*}P < 0.05$ and $^{**}P < 0.01$).

activation of those two genes. However, treatment with SGlc clearly inhibited both MMP-9 and MMP-2 expressions dose-dependently. Moreover at the highest concentration (100 μg/mL) both MMP-2 and MMP-9 expression levels were similar to those in un-stimulated cells. In addition, those effects were compared with those of a well-known MMP inhibitor, doxycycline (DCL) and it had better or similar inhibitory effects compared to SGlc at 10 μg/mL concentration. Gene expressions of MMP-2 and MMP-9 are mediated via two important transcription factors, nuclear factor κB (NF-κB) and activator protein-1 (AP-1). However, it is reported that the promoter of MMP-2 gene lacks the transactivator sequence, AP-1 and gene activation takes place via NF-κB.[7] Therefore that can be presumed to be the reason for low levels of MMP-2 expressions we observed compared to MMP-9.

2.3. SGlc inhibits transcriptional activation of MMPs

To study the effects of SGlc on transcriptional regulation of MMP genes and their transcription factors, specific gene promoters containing pGL3 luciferase reporter vector and β-galactosidase expression vector were co-transfected to HT1080 cells. Then the transfected cells were pre-treated with different concentrations of SGlc and their expressions were stimulated by PMA. As seen in Figure 3a, the relative luciferase activities that represent the levels of MMP-2 and MMP-9 expressions were inhibited by SGlc in a dose-dependent manner. Even though at the lowest concentration (5 μg/mL) of SGlc, inhibition of MMP-9 promoter activity was non-significant, at all the other tested concentrations it exerted a significant ($P < 0.01$) effect on both MMP-2 and MMP-9. However, there was no significant ($P < 0.05$) effect to inhibit promoter activity of MMPs by DCL and that was not in agreement with the results obtained in zymography experiment (Fig. 2). This can be explained due to the suggestion that DCL mainly acts as direct inhibitor of MMPs activity but not as an effective inhibitor of their gene expressions. Therefore, our observations provide clear evidences that SGlc acts as inhibitor of MMP-2 and MMP-9 gene expressions. To further study how these transcriptional down-regulations take place, promoter activities of their transcriptional factors were also studied. According to Figure 3b, promoter activity of NF-κB was clearly inhibited by SGlc following a dose-dependent pattern. Interestingly, SGlc did not have any effect to inhibit promoter activity of AP-1 following stimulation with PMA. Therefore, inhibitory effect of SGlc on MMP-2 and MMP-9 can be explained due to down-regulation of those genes via the transcription factor NF-κB but not AP-1.

2.4. Transcriptional down-regulation of MMPs and NF-κB confirms by their protein level expressions

Western blot studies were carried out to confirm down-regulation of above gene expressions, and their relative protein levels were in agreement with the observed results. As depicted in Figure 4, protein expression levels of both MMP-2 and MMP-9 were dose-dependently inhibited by SGlc treatment. Even though, the protein expression level of MMP-2 was relatively low compared to that of MMP-9, it was better inhibited by SGlc. Moreover, both MMPs were not significantly ($P < 0.05$) inhibited by SGlc at its lowest concentration (5 μg/mL), and it was in line with the results of zymography and reporter gene assay experiments. Since SGlc inhibited promoter activity of NF-κB, its protein level expression was also studied. However, SGlc did not exhibit a clear effect to inhibit AP-1 expression in AP-1 reporter gene assay (Fig. 3b). Therefore, its protein level expression was not assessed in the presence of SGlc. The NF-κB transcription family proteins consist of several protein subunits, and among them p50 and p65 subunits which contain transactivation domains are necessary for gene induction.[8] Therefore, expressions of p50 and p65 NF-κB proteins were studied following

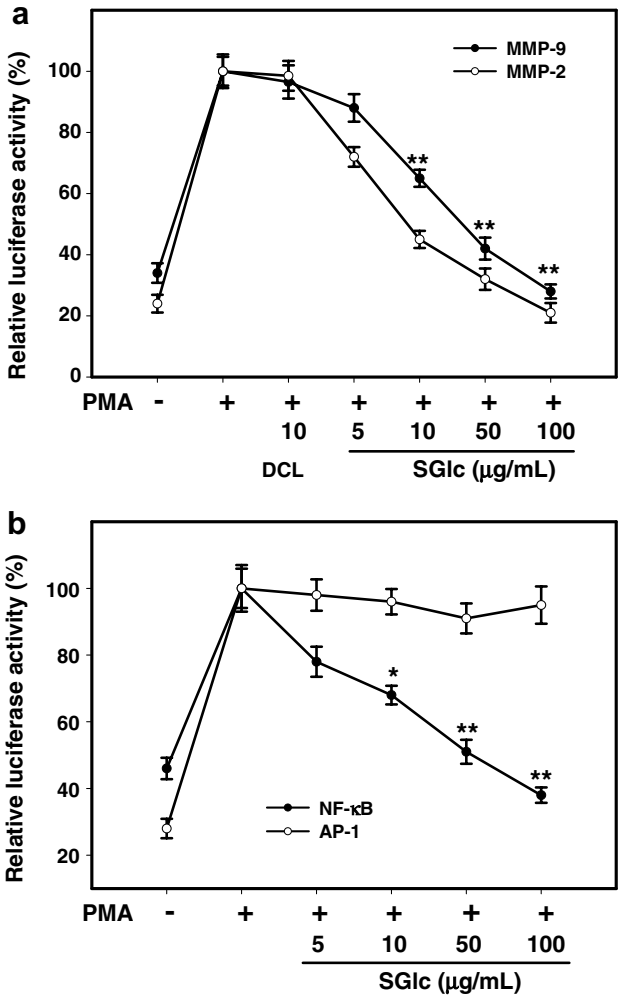

Figure 3. Effects of SGlc on transcriptional activities of MMP-2, MMP-9, and their transcription factors; NF-κB and AP-1. HT1080 cells were transiently co-transfected with target gene promoter containing pGL3 luciferase reporter vector (Promega, Madison, WI) and β-galactosidase expression vector. Following treatment of transfected cells with SGlc, specific gene expressions were determined by relative luciferase activity compared to that of PMA-alone treated cells. Statistical comparisons, $^{*}P < 0.05$ and $^{**}P < 0.01$.

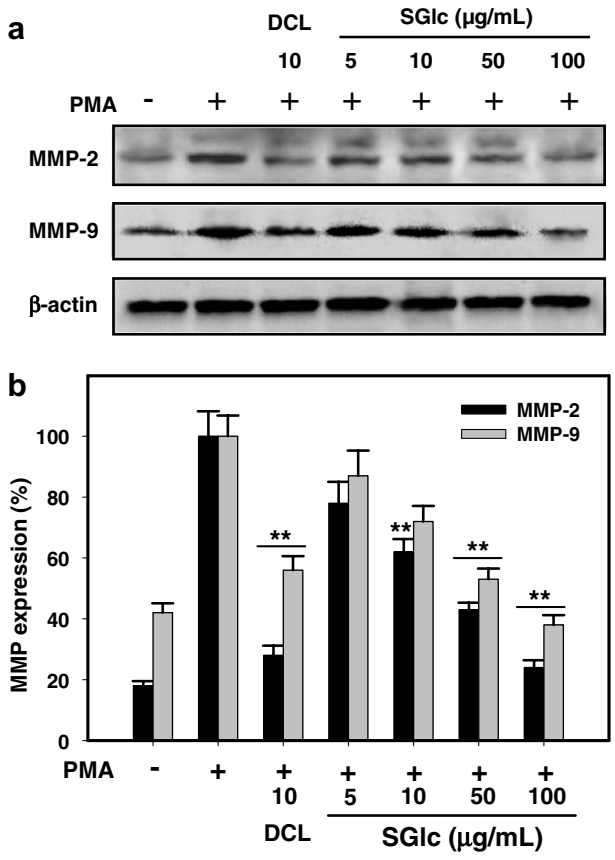

Figure 4. Effect of SGlc on protein expressions of MMP-2 and MMP-9 in HT1080 cells. (a) Cells were pre-treated with different concentrations of SGlc for 1 h and following stimulation with PMA incubation was continued for 24 h. Equal amounts of protein in the cell lysates were electrophoresed and levels of above protein expressions were determined using specific antibodies. Respective protein levels of β-actin were used to confirm the equal mounts of protein used for electrophoresis. (b) Areas and intensities of protein bands were determined by densitometry and expressed as a percentage MMP expression compared to protein levels of PMA-alone treated cells. Statistical comparisons, $^{*}P < 0.05$ and $^{**}P < 0.01$.

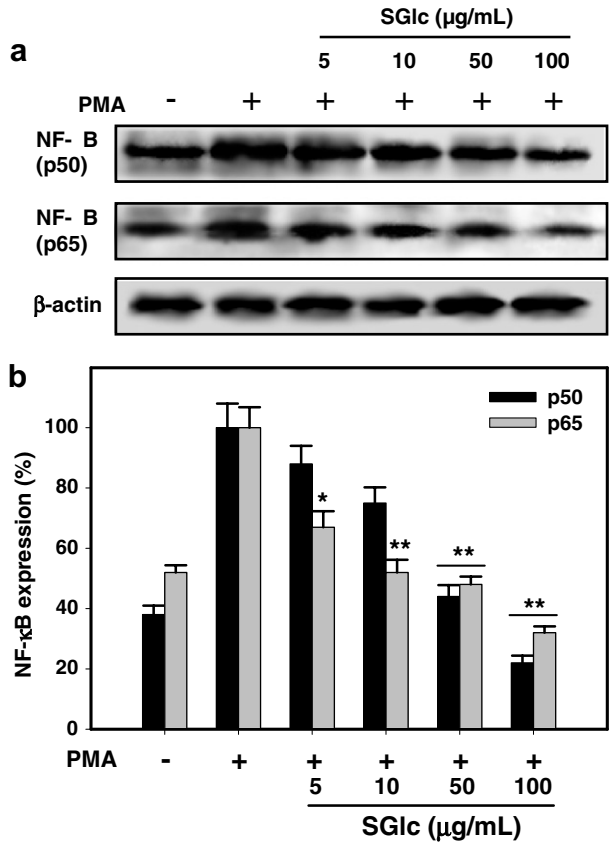

Figure 5. Inhibition of NF-κB protein expressions by SGlc. (a) HT1080 cells were pre-treated with different concentrations of SGlc for 1 h, stimulated with PMA, and followed by 24 h of incubation. Equal amounts of protein in the cell lysates were electrophoresed and levels of NF-κB (p50) and NF-κB (p65) protein expressions were determined using specific antibodies. Respective protein levels of β-actin were used to confirm the equal mounts of protein used for electrophoresis. (b) Areas and intensities of protein bands were determined by densitometry and expressed as a percentage NF-κB expression compared to protein levels of PMA-alone treated cells. Statistical comparisons, $^{*}P < 0.05$ and $^{**}P < 0.01$.

treatment with SGlc. As shown in Figure 5, both proteins were inhibited by SGlc in a more or less similar manner. However, at low concentrations inhibition of p65 protein expression was more effective than that of p50. Inhibitors of MMP-2 and MMP-9 reported so far have different capacities to inhibit NF-κB and AP-1 transcription factors. Only a few reports on the promoters of MMP-9 and MMP-2 suggest the higher involvement of NF-κB for the activation of MMPs than AP-1. But the relative involvement of NF-κB and AP-1 for the expression of MMP-2 and MMP-9 has not been clearly confirmed. Therefore, it is hard to give a specific comment on the importance of inhibiting only NF-κB but not AP-1 by SGlc.

Summarizing the results of this study, it can be concluded that SGlc acts as an inhibitor of MMP-2 and MMP-9 expressions followed by down-regulation of NF-κB. Further, it can be presumed that inhibition of MMP-2 and MMP-9 can be one of the mechanisms of SGlc to be an effective slow-acting drug in relieving the symptoms of OA.

3. Experimental

3.1. Materials

Chitosan used for the preparation of glucosamine (Glc) was kindly donated by Kitto. Life Co. (Seoul, Korea). All chemicals required for synthesis, including SO_3-pyidine, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Human fibrosarcoma cell line (HT1080) and normal human fibroblasts (MRC-5) were obtained from American Type of Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), Trypsin-EDTA, penicillin/streptomycin, fetal bovine serum (FBS), and other materials required for culturing cells were purchased from Gibco BRL, Life Technologies (USA). Primary and secondary antibodies used for Western blot analysis were

purchased from Santa Cruz Biotechnology Inc. (CA, USA) and Amersham Pharmacia Biosciences (NJ, USA), respectively. NF-κB and AP-1 gene promoter reporter vectors were purchased from Clontech (CA, USA). MMP-2 and MMP-9 gene promoter reporter vectors were kindly donated by Dr. Sang-Oh Yoon (KAIST, Taejeon, Korea).

3.2. Synthesis and structure identification of SGlc

Chitosan was hydrolyzed with concentrated HCl for 3 h and the resultant Glc was precipitated with 100% ethanol. Sulfation of Glc was carried out with SO_3-pyidine complex according to a method described previously.[9] The structure of purified SGlc was determined by ^1H NMR, ^{13}C NMR spectroscopy (JNM-ECP-400 NMR spectrometer, JEOL, Japan), FT-IR spectroscopy (Spectrum 2000 FT-IR spectrophotometer, Perkin–Elmer, USA), and elemental (C, N, and H) analysis (Elementar Analysesysteme, Elementar Vario, EL, USA).

SGlc: FT-IR (KBr) ν_{max} 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) cm^{-1}[10]; ^{13}C NMR (D_2O, 400 MHz) δ: 52.1 (C-2), 67 (C-6), 69, 76, and 72 (C-3, 4, 5), 89, 92 (C-1);[11] elemental analysis: S% (11.13), C% (24.98), N% (5.04), H% (4.4).

3.3. Cell culture

Human fibrosarcoma cells (HT1080) were cultured as monolayers in DMEM containing 10% fetal bovine serum, 2 mM glutamine, and 100 μg/mL penicillin–streptomycin at 5% CO_2 and 37 °C humidified atmosphere. For experiments, cells were passaged at least for 5times and detached with Trypsin-EDTA.

3.4. Determination of cell viability

To determine cytocompatible effects of SGlc, HT1080 cells were seeded into 96-well plates at a density of about 1×10^4 cells/well and incubated with different concentrations of SGlc for 24 h in the presence or absence of serum. After incubation, 100 μl of MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) reagent (0.5 mg/ml final concentration) was added to each well and incubation was continued for another 4 h. Mitochondrial succinate dehydrogenase in live cells converts MTT into visible formazan crystals during incubation. The formazan crystals were then solubilized in DMSO and the optical density was measured at 540 nm by using UV microplate reader (Tecan Austria GmbH, Austria).[12] Relative cell viability was calculated compared to the non-treated blank group. The data were expressed as means of at least three independent experiments and $P < 0.05$ was considered significant.

3.5. Gelatin zymography

Gelatin zymography was used to determine expression and activities of MMP-2 and MMP-9 in SGlc treated HT1080 as described previously.[13] For that, HT1080 cells were seeded in 24-well plates using serum-free media and pre-treated with different concentrations of SGlc for 1 h. Expression of MMPs was stimulated by treating with PMA (10 ng/mL) and incubation was continued for another 40 h. After incubation, conditioned media were collected and their protein contents were determined by Bradford protein determination method.[14] After normalizing the protein content equal amounts of proteins were electrophoresed under non-reducing conditions on 10% polyacrylamide gels containing 1.5 mg/mL gelatin. Following electrophoresis, polyacrylamide gels were washed with 50 mM Tris–HCl (pH 7.5) containing 2.5% Triton X-100 to remove sodium dodecyl sulfate. Gels were then incubated overnight at 37 °C in a developing buffer containing 10 mM $CaCl_2$, 50 mM Tris–HCl, and 150 mM NaCl to digest gelatin by MMPs. Areas of gelatin hydrolyzed by MMPs were visualized as clear zones against blue background by Coomassie blue staining and the intensities of the bands were estimated by densitometry (Multi Gauge V3.0 software, Fujifilm Life Science, Tokyo, Japan).

3.6. Transfection and reporter gene assay

In separate experiments, HT1080 cells were transiently co-transfected with β-galactosidase expression vector together with MMP-2, MMP-9, NF-κB, or AP-1 promoter binding site-luciferase reporter vector at a time using Lipofectamine™ 2000 reagent (Invitrogen, USA). Transfected cells were treated with different concentrations of SGlc for 24 h, washed once with cold PBS, and then lysed with hypertonic lysis buffer (25 mM Tris–HCl, pH 8.0, containing 2 mM DDT and 1% Triton X-100). Equal amounts (20 μL) of cell lysates and luciferase substrate (Promega, USA) were mixed in a 96-well plate and luminescence intensity was measured with a luminescence microplate reader (Tecan Austria GmbH, Austria). Then luciferase activity was normalized to transfection efficiency monitored by β-galactosidase expression vector in ONPG buffer. The level of reporter gene expression was determined as a ratio, compared with cells stimulated by PMA (10 ng/mL) alone.

Transfected cells were visualized by X-Gal staining method as described previously with modifications.[15] Briefly, transfected cells were fixed with 0.5% glutaraldehyde and stained with X-Gal solution containing 20 mM $K_3Fe(CN)_6$, $K_4Fe(CN)_6$, and 1 mM $MgCl_2$. After 24 h of incubation at 37 °C, transfected cells were visualized with blue color under a light microscope.

3.7. Western blotting

Western blotting was performed according to standard procedures. HT1080 cells treated with different concentrations of SGlc and followed by stimulation with PMA were lysed in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 0.4% Nonidet P-40, 120 mM NaCl, 1.5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 80 μg/mL leupeptin, 3 mM NaF, and 1 mM DTT at 4 °C for 30 min. Cell lysates (about 10 μg of total

proteins) were resolved on a 4–20% Novex® gradient gel (Invitrogen, USA), electrotransferred onto a nitrocellulose membrane, and blocked with 10% skim milk. Specific primary antibodies (Santa Cruz Biotechnology Inc., CA, USA) and their secondary antibodies were used to detect respective proteins using chemiluminescent ECL assay kit (Amersham Pharmacia Biosciences, NJ, USA) according to the manufacturer's instructions. Protein bands were visualized using LAS3000® Luminescent image analyzer and protein expression was quantified by Multi Gauge V3.0 software (Fujifilm Life Science, Tokyo, Japan).

3.8. Data analysis

One-way analysis of variance was used for all statistical analyses using independent experiments and data are represented as means ± SEM. Individual values were compared by Dunnett's test and $P < 0.05$ considered as significant unless otherwise stated.

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