Original article

Effect of glycated human serum albumin on BMP2-induced osteoblastgenesis in C2C12 cells

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Abstract

Aim: Bone remodeling by which mature bone tissue is removed by osteoclast and new bone tissue is formed by osteoblast, is important to maintain bone mass and quality. Imbalanced bone homeostasis increases fracture risks and lead to osteoporosis. Accumulation of advanced glycation end products (AGEs) plays a role in diabetic complications and patients with diabetes mellitus have a higher incidence rate of osteoporosis than healthy subjects. In this research, we investigated the effect of AGEs on osteoblast differentiation (osteoblastgenesis).

Methods: For osteoblstgenesis, mouse myoblast C2C12 cells were stimulated with bone morphogenetic protein 2 (BMP2). To determine the effect of AGEs on osteoblastgenesis, cells were treated with BMP2 with or without AGEs, which were formed by glyceraldehyde and human serum albumin (HSA-glycer), and we performed quantitative real-time PCR analysis against alkarine phosphatase (ALP) and osteocalcin (OC), marker of osteoblastgenesis. We further investigated the effect of AGEs on the expression of runt related transcription factor 2 (Runx2) and osterix, major transcription factors of osteoblastgenesis. To see the effect of AGEs on Smad pathway, which partially regulates the BMP2 signaling pathway, western blot analyses against phosphorylation of Smad 1/5/9 were examined. Finally, to evaluate the effect of AGEs on bone turnover, we investigated the ratio of osteoprotegerin (OPG) and receptor activator of NF-κB ligand (RANKL), a major indicator of bone turnover.

Results: HSA-glycer inhibited BMP2-induced expression of ALP and OC. The expression of transcription factors and phosphorylation of Smad 1/5/9 were partially suppressed by HSA-glycer. Decreasing RANKL/OPG ratios by HSA-glycer suggests that AGEs are likely to delay bone turnover.

Conclusion: Our findings indicate that AGEs may inhibit BMP2-induced osteoblastgenesis via alteration of the Smad pathway.

KEY WORDS: glycative stress, AGEs, bone morphogenetic protein 2, osteoblastgenesis, osteoporosis, bone turnover

Introduction

Osteoporosis is a disease which is characterized by low bone mass and deterioration of the quality of bone tissue [1]. In Japan, over twelve million people suffered from osteoporosis in 2015 [2] and patients faced fracture risks, leading to the loss of their quality of life. While Osteoporosis can affect people at almost any age, it is most common among those 50 years of age or older, especially postmenopausal women [3, 4]. Other than the age, Schwartz *et al*. demonstrated that older women with type 2 diabetes have higher fracture rates than non-diabetic women [5, 6]. Both bone mass (or bone mineral density, BMD) and bone quality are important for bone strength, however, meta analyses found that type 2 diabetic patients have normal or high BMD compared to healthy subjects [7]. Thus, plenty of studies have been conducted to clarify the contribution of diabetes toward the loss of bone quality.

Diabetes mellitus is a chronic symptom in which the levels of glucose in the blood are too high. Excess blood sugar reacts with proteins non-enzymatically and forms advanced glycation end products (AGEs). Several lines of evidence have shown that the accumulation of AGEs is implicated in multiple

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diseases [8-13], including osteoporosis [14]. Recently, Takeuchi *et al*. demonstrated that glyceraldehyde, which is synthesized in fructolysis, formed more highly toxic AGEs than glucose [15]. To maintain the mechanical properties of bones, type I collagen which is a major protein in bone tissue form the enzymatic cross-link as a post-transcriptional modification [16]. While, patients with osteoporosis and diabetes mellitus, AGEs play a role for forming dispensable cross-link in bone collagen, leading to the loss of bone resilience [17]. Moreover, Shiraki M. *et al*. reported that pentosidine, which is a fluorescent cross-linking type of AGEs, is an independent risk factor for osteoporotic incidence of vertebral fractures [18]. These data indicate that accumulation of AGEs may be involved in the loss of bone quality.

Bone remodeling is also important to maintain bone homeostasis. Mature bone tissue is removed by osteoclast and new bone tissue is formed by osteoblast, and about 10% of bone material is renewed each year [19]. The imbalance between these two processes results in significant bone loss and decreased bone quality. In our previous report, we demonstrated that AGEs, made from human serum albumin (HSA) and glyceraldehyde, inhibited the receptor activator of nuclear factor kappa-B ligand (RANKL)-induced osteoclast differentiation (osteoclastgenesis) in RAW264.7 cells [20]. Except for bone absorption, mature osteoclasts secrete cytokines which regulate osteoblast differentiation (osteoblastgenesis) such as transforming growth factor beta (TGF-β) and bone morphogenetic protein 2 (BMP2) [21], thus, our previous data suggest that AGEs may delay bone remodeling via inhibition both osteoclastgenesis and osteoblastgenesis, leading to loss of bone quality.

BMP belongs to the large super family of TGF-ß and BMPs, including BMP2, are well known to be implicated in osteoblastgenesis [22]. BMPs induce osteoblastgenesis through both Smad-dependent or –independent signal pathways [23]. In Smad dependent pathways, when the secreted BMP2 binds to type II receptor, type I receptor kinase is phosphorylated by the activated type II receptor kinase, and then, the signaling transduces to Smad proteins. Phosphorylated Smad 1/5/8 regulate expression of osteoblast specific proteins such as alkaline phosphatase (ALP) and osteocalcin (OC) and the osteogenic transcription factor such as Runx2, osterix and Dlx5 [24-26].

Several studies have been demonstrated that AGEs induced apoptosis in osteoblast cells via oxidative stress [27-29] and McCarthy *et al*. reported that AGEs made from serum bovine albumin (BSA) and glucose (AGE-BSA) inhibited cell proliferation and ALP activity in two different lines of osteoblast-like cells after several days incubation [30]. In the present study, we are focusing on the effect of AGEs on BMP2-induced osteoblastgenesis without cytotoxicity to clarify if AGEs alter osteoblastgenesis independent of cell damage.

Materials and Methods
Materials

BMP2 Human/Rat/Mouse recombinant protein was obtained from R&D systems (Minneapolis, MN, USA).

Human serum albumin (HSA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were obtained from Wako (Osaka, Japan) as analytical grade.

Cell culture

C2C12 cells, mouse myoblast, were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Dulbecco's Modified Eagle Medium with high glucose (DMEM, Wako) containing 10% fetal bovine serum (Sigma-Aldrich) and antibiotics (Wako), and grown at 37°C under an atmosphere of 5% CO_2.

Preparation of glycated proteins

Glycated proteins were made by human serum albumin (HSA) and glyceraldehyde. HSA (8 mg/mL) and 33 mmol/L glyceraldehyde in 50 mmol/L phosphate buffer (PB, pH 7.4) were incubated at 60°C for 40 h. We also prepared heated HSA, by which HSA without glyceraldehyde were incubated at 60°C for 40 h as a control. To remove remaining unreacted glycating agents, reaction mixtures were ultrafiltrated using centrifugal filter units (10K, Merck Millipore Burlington, MS, USA) and were washed three times using distilled water.

Determination of Cell viability

To determine the cell viability, a WST-8 assay was performed using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. Data were expressed as percentage of water or vehicle control treated cells.

RNA extraction and Quantitative real-time PCR analysis

C2C12 cells were seeded at 4 x 10^3 cells/well in a 24-well plate. Twenty-four hours later, cells were stimulated with BMP2, glycated HSA for indicated concentration and time period. Then, cells were washed by PBS once, then, 400 μL ISOGEN II (Nippon Gene, Tokyo, Japan) were used for RNA extraction according to the manufacturer's protocol. Amount of RNA was measured by Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA USA). A five-hundred ng total RNA was reverse-transcribed with PrimeScript™ RT Master Mix (Takara Bio Inc., Shiga, Japan) using Applied Biosystems 2720 Thermal cycler (Thermo Fisher Scientific, MA). Quantitative real-time PCR (qPCR) was performed with a Thunderbird™ SYBR qPCR mix (Toyobo Co., Osaka, Japan) according to the manufacturer's protocol with gene-specific primers (Thermo Fisher Scientific). The primers were used are as follows: Alkaline phosphatase (ALP), 5′-GAT CAT TCC CAC GTT TTC AC-3′ (forward), 5′-TGC GGG CTT GTG GGA CCT GC-3′ (reverse); Osteocalcin (OC), 5′- CCT CTC GAC CCG ACT GCA GAT C -3′ (forward), 5′-AGC TGC AAG CTC TCT GTA ACC ATG AC -3′ (reverse); Osterix, 5′- CGT CCT CTC TGC TTG AGG AA -3′ (forward), 5′- GGG CTG AAA GGT CAG CGT AT -3′ (reverse); Runx2, 5′- CAG TCC CAA CTT CCT GTG CT -3′ (forward), 5′- CCC ATC TGG TAC CTC TCC GA -3′ (reverse); GAPDH, 5′- TGA AGG TCG

GTG TGA ACG GAT TGG C -3′(forward), 5′ - CAT GTA GGC CAT GAG GTC CAC CAC -3′ (reverse); Receptor activator of NFκB ligand (RANKL), 5′- AGC CAT TTG CAC ACC TCA CC -3′(forward), 5′-AAG CAA ATG ATT GGC GTA CAG G -3′(reverse); Osteoprotegerin (OPG), 5′- AGT GTG AGG AAG GGC GTT AC -3′(forward), 5′- AAT GTG CTG CAG TTC GTG TG -3′ (reverse).

ALP staining

C2C12 cells were seeded at 8×10^3 cells/well in a 12-well plate. Twenty-four hours later, cells were stimulated with or without 400 ng/mL BMP2 and glycated HSA for 72 h. To examine the alkaline phosphatase activity, Alkaline Phosphatase Staining Kit (Cosmo Bio Ltd. Tokyo, Japan) was used as according to the manufacturer's protocol. Briefly, cells were washed with PBS three times, then, fixed with 10% formaldehyde for 10 min at room temperature. After washing with distilled water, the cells were incubated with ALP activity solution for 20 min at 37°C. The cell images were captured by inverted microscope (CKX41, OLYMPUS corp. Tokyo, Japan) with digital camera (DP21, OLYMPUS corp.) with Cell Sense software (OLYMPUS corp.).

Western blot analysis

C2C12 cells were seeded in a 12-well plate at a density of 5×10^4 cells/well and incubated for 24 h. Then, they were treated with or without 400 ng/mL BMP2, glycated or heated-HSA for the indicated time periods. The entire cell lysate was extracted with RIPA buffer containing 50 mmol/L Tris-HC1 (pH 7.4), 150 mmol/L NaC1, 0.1% SDS, 1% Triton X-100 with complete protease inhibitor (Wako) and phosphatase inhibitor (Roche Applied Science, Penzberg, Germany). Cell lysates were electrophoresed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (8% polyacrylamide), then proteins were transferred to a polyvinylidene difluoride (PVDF) membrane followed by blocking with a 5% skim milk solution in TBS-T. Then, the membranes were immunoblotted with each primary antibody. The antibodies against Runx2, phospho-Smad 1/5/9 and Smad 1 were from cell signaling technology (Danvers, MA, USA), anti-GAPDH was purchased from Abcam (Cambridge, UK), and anti-β-actin was obtained from Sigma-Aldrich. The antigen-antibody complexes were visualized with the appropriate secondary antibodies (Santa Cruz Biotechnology, CA, USA) and chemiluminescence horseradish peroxidase (HRP) substrate along with detection system, as recommended by the manufacturer. The results illustrated in each figure are representative of three independent experiments. Image J was used to measure the optical density of the protein bands.

Statistics.

The statistical analysis was performed by Tukey-Kramer's multiple comparison test. Differences were considered significant at p values less than 0.05.

Results

Osteoblastgenesis were induced by BMP2 in time-and dose-dependent manner in C2C12 cells.

First of all, we estimated the condition of osteoblastgenesis in C2C12 cells. Cells were stimulated with 400 ng/mL BMP2 for the indicated time periods and we performed qPCR analyses (**Fig. 1-a**). Both mRNA levels of alkarine phosphatase (ALP), early markers of osteoblastgenesis and osteocalcin (OC), and markers of mature osteoblasts were induced in a time-dependent manner. Next, C2C12 cells were incubated with an indicated concentration of BMP2 for 72 h, then, qPCR analyses were conducted. Both mRNA levels of ALP and OC were induced in a dose-dependent manner (**Fig.1-b**). Especially over 300 ng/mL treatment, BMP2 induced those mRNA levels significantly. Furthermore, to examine the effect of BMP2 on ALP activity in C2C12 cells, we performed an ALP staining assay. As shown in **Fig. 1-c**, ALP activity was induced by 400 ng/mL BMP2 for 72 h. Thus, to investigate the effect of glycated proteins on osteoblastgenesis, C2C12 cells were stimulated with 400 ng/mL BMP2 for 72 h.

The effect of glycated HSA on BMP2-induced osteoblastgenesis.

Initially, we evaluated the cytotoxicity of glycated proteins using WST-8 assay. Seventy-two hours treatment of glyceraldehyde-derived glycated HSA (HSA-glycer) did not induce cell death up to 400 μg/mL (**Fig. 2-a**). Next, to investigate the effect of HSA-glycer on osteoblastegenesis, we treated with BMP2 and HSA-glycer at the same time in C2C12 cells. Cells were treated with 400 ng/mL BMP2 and indicated concentration of HSA-glycer for 72 h, then, we performed qPCR analyses. Both mRNA levels of BMP2-induced ALP and OC were inhibited by HSA-glycer (**Fig. 2-b**). We also performed ALP staining assay, HSA-glycer inhibited BMP2-induced ALP activity (**Fig.2-c**). These data indicated that HSA-glycer mediate BMP2-induced osteoblastgenesis.

Glycated HSA altered the expression of transcription factors that regulate osteoblastgenesis.

Several major transcription factors that regulate osteoblastgenesis have been identified, such as runt related transcription factor 2 (Runx2) and osterix [25]. In this experiment, we investigated whether HSA-glycer mediate the expression levels of Runx2 and osterix. As shown in **Fig. 3-a**, 400 ng/mL BMP2 induced mRNA levels of osterix and it was inhibited by 400 μg/mL HSA-glycer. HSA-heated, by which HSA were incubated at 60°C for 40 h without glyceraldehyde, did not affect BMP2-induced mRNA expression of osterix. Also, BMP2-induced mRNA levels of Runx2 were inhibited by HSA-glycer but not HSA-heated (**Fig. 3-b**). We further determined the protein levels of Runx2 and it was also inhibited by HSA-glycer, but not HSA-heated (**Fig. 3-c**). These data indicated that HSA-glycer affect the expression of transcription factors which regulate osteoblastgenesis.
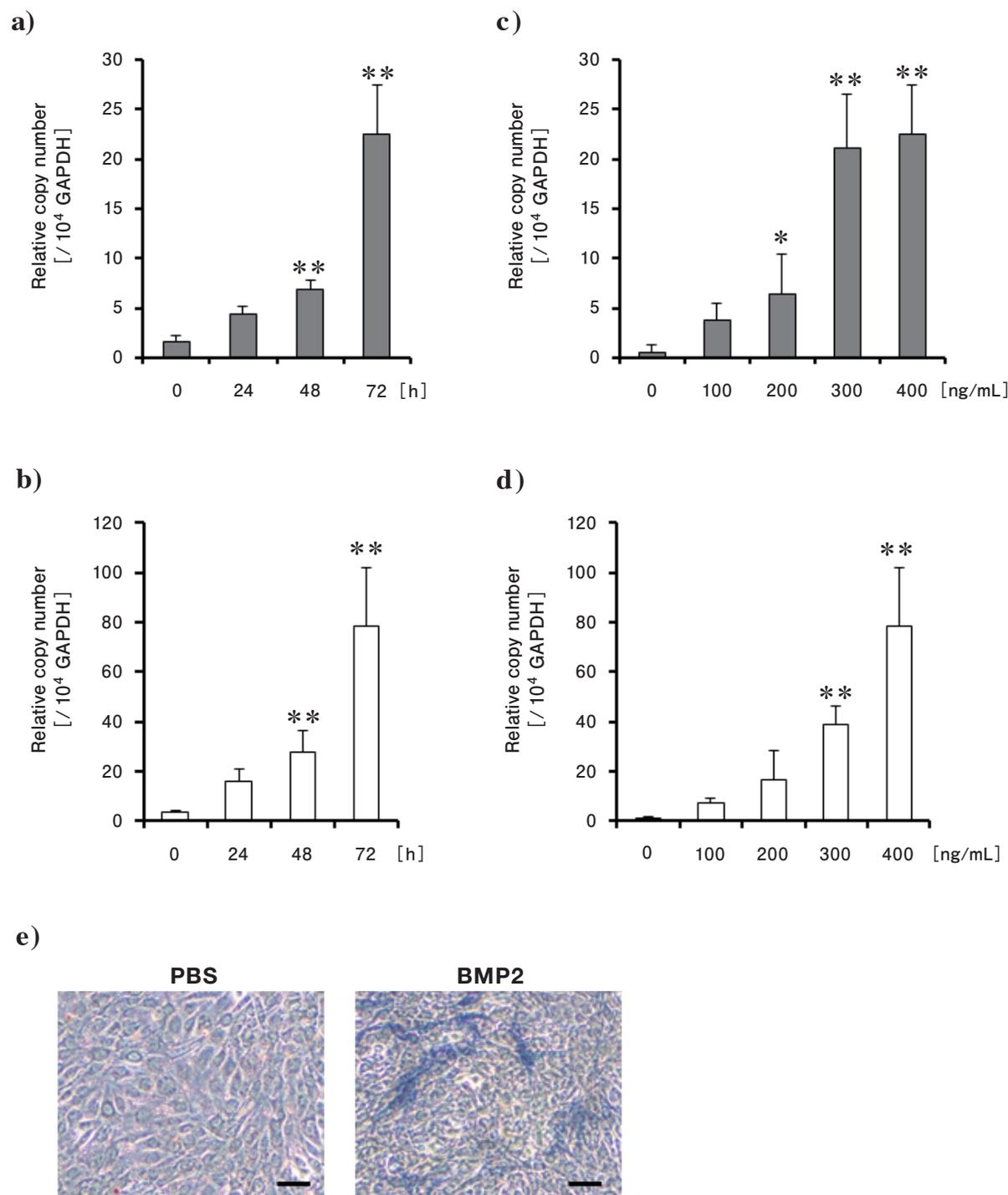
Fig. 1. BMP2 increases the expression levels of ALP and OC in C2C12 cells.

(**a, b**) Sucrose (Suc). Time-dependent induction of ALP and OC, the markers of osteoblastgenesis, by BMP2. C2C12 cells were treated with 400 ng/mL BMP2 for up to 72 h. Quantitative real-time PCR (qPCR) analyses were performed for (**a**) ALP and (**b**) OC. All data obtained were normalized by GAPDH and shown as the mean ± SD (n = 6). ** $p < 0.01$ vs. Time 0. (**c, d**) Concentration-dependent induction of ALP and OC by BMP2. C2C12 cells were treated with up to 400 ng/mL BMP2 for 72 h. The expression levels of (**c**) ALP and (**d**) OC. All data obtained were normalized by GAPDH and shown as the mean ± SD (n = 6). * $p < 0.05$, ** $p < 0.01$ vs. 0 ng/mL BMP2. (**e**) BMP2 induced ALP activity in C2C12 cells. C2C12 cells were treated with 400 ng/mL BMP2 for 72 h. ALP staining were performed to determine ALP activity. The images shown are representative of three independent experiments with similar results. Bar, 100 μm. BMP2, bone morphogenetic protein 2; ALP, alkaline phosphatase; OC, osteocalcin; PCR, polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SD, standard deviation.
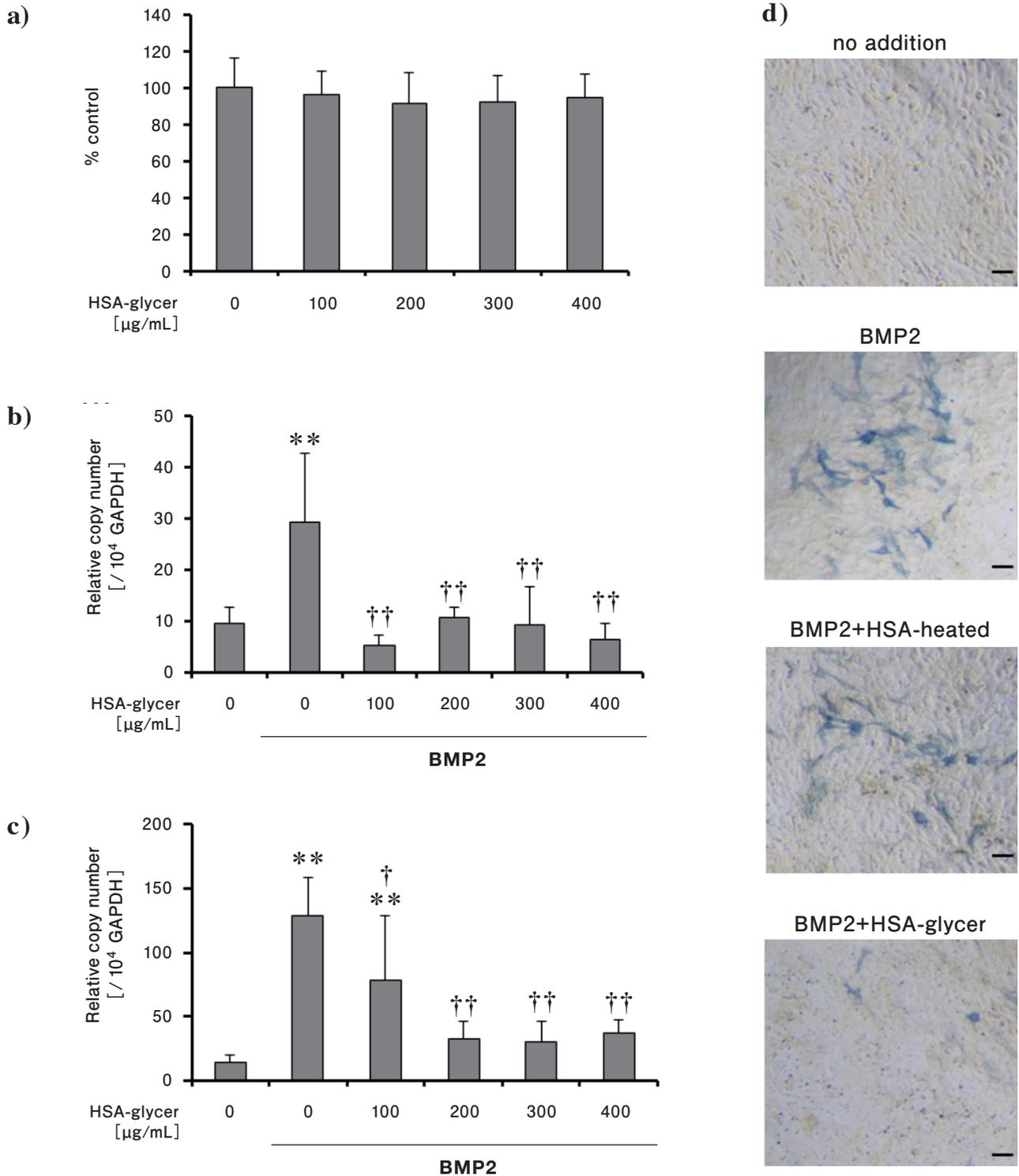
a)

b)

c)

d)

no addition

BMP2

BMP2+HSA-heated

BMP2+HSA-glycer

***Fig. 2.* Glyceraldehyde-derived glycated HSA suppress BMP2-induced ALP and OC in C2C12 cells.**

(a, b, c) C2C12 cells were treated with glyceraldehyde-derived glycated HSA (HSA-glycer) up to 400 μg/mL for 72 h with or without 400 ng/mL BMP2. **(a)** WST-8 assay was performed to determine cytotoxicity of HSA-glycer (n = 6). **(b, c)** Effect of HSA-glycer on BMP-2-induced mRNA expression of **(b)** ALP and **(c)** OC. All data obtained were normalized by GAPDH and shown as the mean ± SD (n=6). ** $p < 0.01$ vs. without BMP2. † $p < 0.05$, †† $p < 0.01$ vs. BMP2 without HSA-glycer. **(d)** Effect of glycated HSA on BMP-2-induced ALP activity. C2C12 cells were treated with 400 μg/mL HSA-glycer or HSA-heated and 400 ng/mL BMP2 for 72 h. ALP activity was examined by ALP staining assay. The images shown are representative of three independent experiments with similar results. Bar, 100 μm. HSA, human serum albumin; BMP2, bone morphogenetic protein 2; ALP, alkaline phosphatase; OC, osteocalcin; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SD, standard deviation.
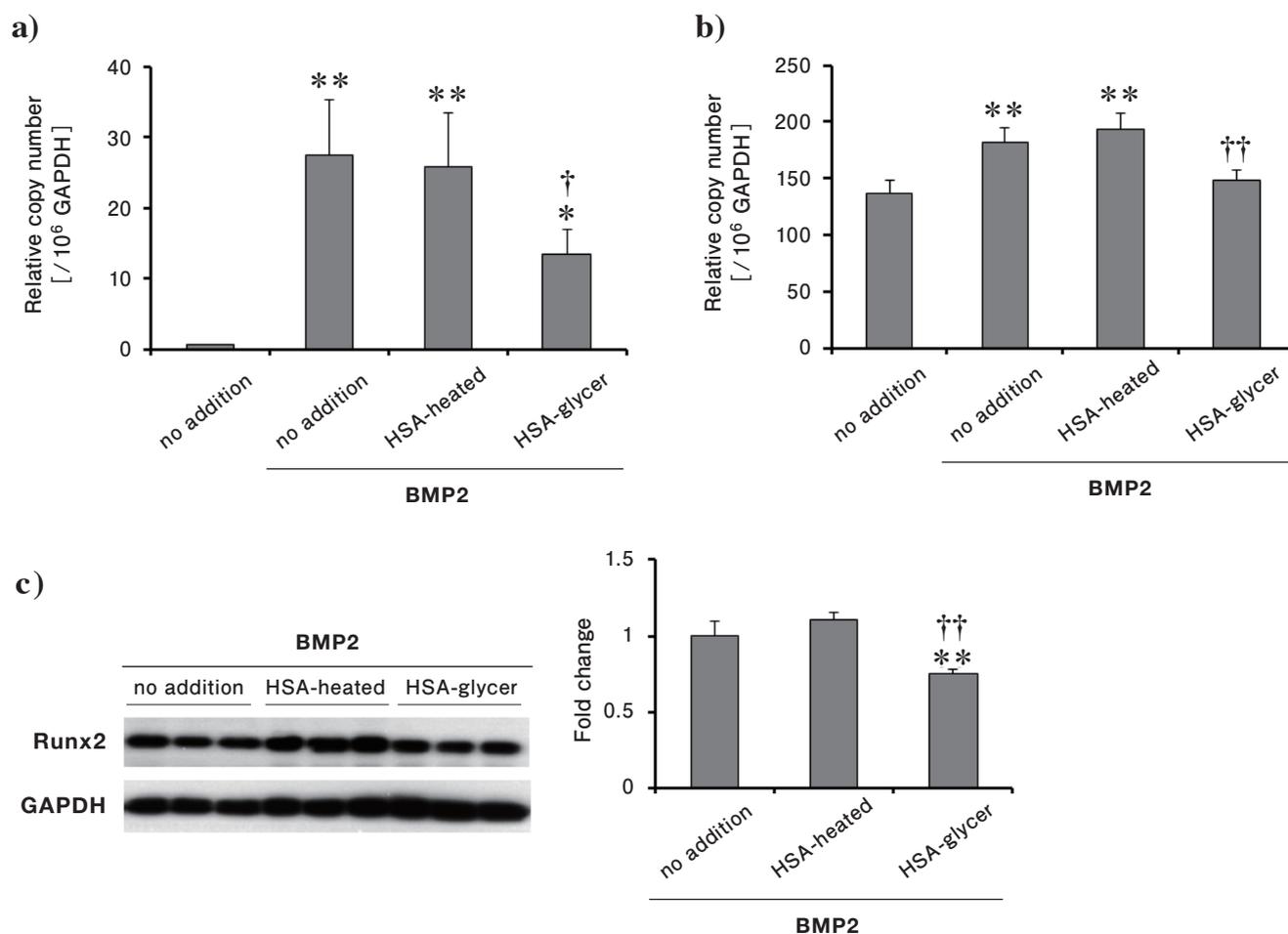
a)

b)

c)

Fig. 3. **Glyceraldehyde-derived glycated HSA inhibit the expression of transcription factors regulate osteoblastgenesis.**

C2C12 cells were treated with 400 µg/mL HSA-glycer or HSA-heated and 400 ng/mL BMP2 for 48 h. qPCR analysis were performed for **(a)** Osterix and **(b)** Runx2. All data obtained were normalized by GAPDH and shown as the mean ± SD (n = 4). * p < 0.05, ** p < 0.01 vs. no addition. † p < 0.05, †† p < 0.01 vs. BMP2. **(c)** Samples (30 µg of proteins) of the crude extract were used for western blot analysis using antibody against Runx2. The bars show the mean ± SD (n = 3) of the ratio against BMP2. ** p < 0.01 vs. BMP2. †† p < 0.01 vs. BMP2 with HSA-heated. HSA, human serum albumin; glycer, glyceraldehyde; BMP2, bone morphogenetic protein 2; PCR, polymerase chain reaction; qPCR, quantitative real-time PCR; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SD, standard deviation.

Glycated HSA inhibited BMP2-induced phosphorylation of Smad in C2C12 cells.

The BMP2 signaling pathway is partially regulated by the Smad pathway [31]. In canonical BMP signaling cascade, BMP receptor I and II form dimer and type II receptor phosphorylates and activates the type I receptor. Then, the type I receptor phosphorylates Smad 1/5/8 (Smad 8 is also known as Smad 9). In this experiment, we investigated the effect of HSA-glycer on BMP2-induced Smad pathway. Twenty-four hours treatment of 400 ng/mL BMP2 induced phosphorylation of Smad 1/5/9 and it was significantly inhibited by co-treatment of 400 µg/mL HSA-glycer (**Fig. 4**). These data indicate that AGEs, which were contained in HSA-glycer, may affect early stages of osteoblastgenesis.

Effect of glycated proteins on BMP2-induced cytokines regulate bone remodeling.

Our data suggests that HSA-glycer inhibits osteoblastgenesis. When we argue the bone remodeling, osteoclastgenesis is also a key incident. Osteoblasts secrete osteoprotegerin (OPG) and receptor activator of NF-κB ligand (RANKL) which are cytokines that play an important role for the osteoclastgenesis. The binding of RANKL to its receptor RANK in osteoclast precursors cells triggers the occurrence of osteoclastgenesis, and OPG acts as a decoy receptor, preventing the binding of RANKL on RANK. Because of the opposite effects of OPG and RANKL on osteoclastgenesis, the RANKL/OPG ratio is a major indicator of bone turnover. We examined qPCR analyses to determine the effect of glycated HSA on the expression levels of OPG and RANKL. Cells were treated with 400 ng/mL BMP2 and 400 µg/mL HSA-glycer for 48 h, then qPCR analysis were performed. As shown in **Figs. 5-a** and b, HSA-glycer down-regulated the mRNA levels of RANKL (**Fig. 5-a**), but not OPG (**Fig. 5-b**). Thus, HSA-glycer decreased RANKL/OPG ratios means HSA-glycer may also delay osteoclastgenesis.
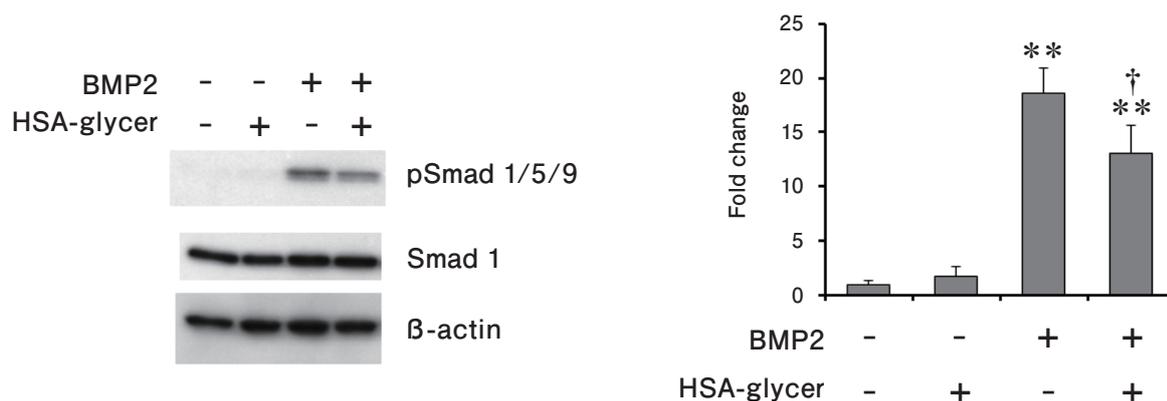
Fig. 4. Effect of glyceraldehyde-derived glycated HSA on BMP2-induced Smad phosphorylation.

C2C12 cells were treated with 400 µg/mL HSA-glycer and 400 ng/mL BMP2 for 24 h. Samples (20 µg of proteins) of the crude extract were used for western blot analysis using antibody against phosphor-Smad 1/5/9, Smad 1 and β-actin. The bars show the mean ± SD (n = 3) of the ratio against no addition. ** $p < 0.01$ vs. no addition. † $p < 0.05$ vs. BMP2. HSA, human serum albumin; glycer, glyceraldehyde; BMP2, bone morphogenetic protein 2; SD, standard deviation.
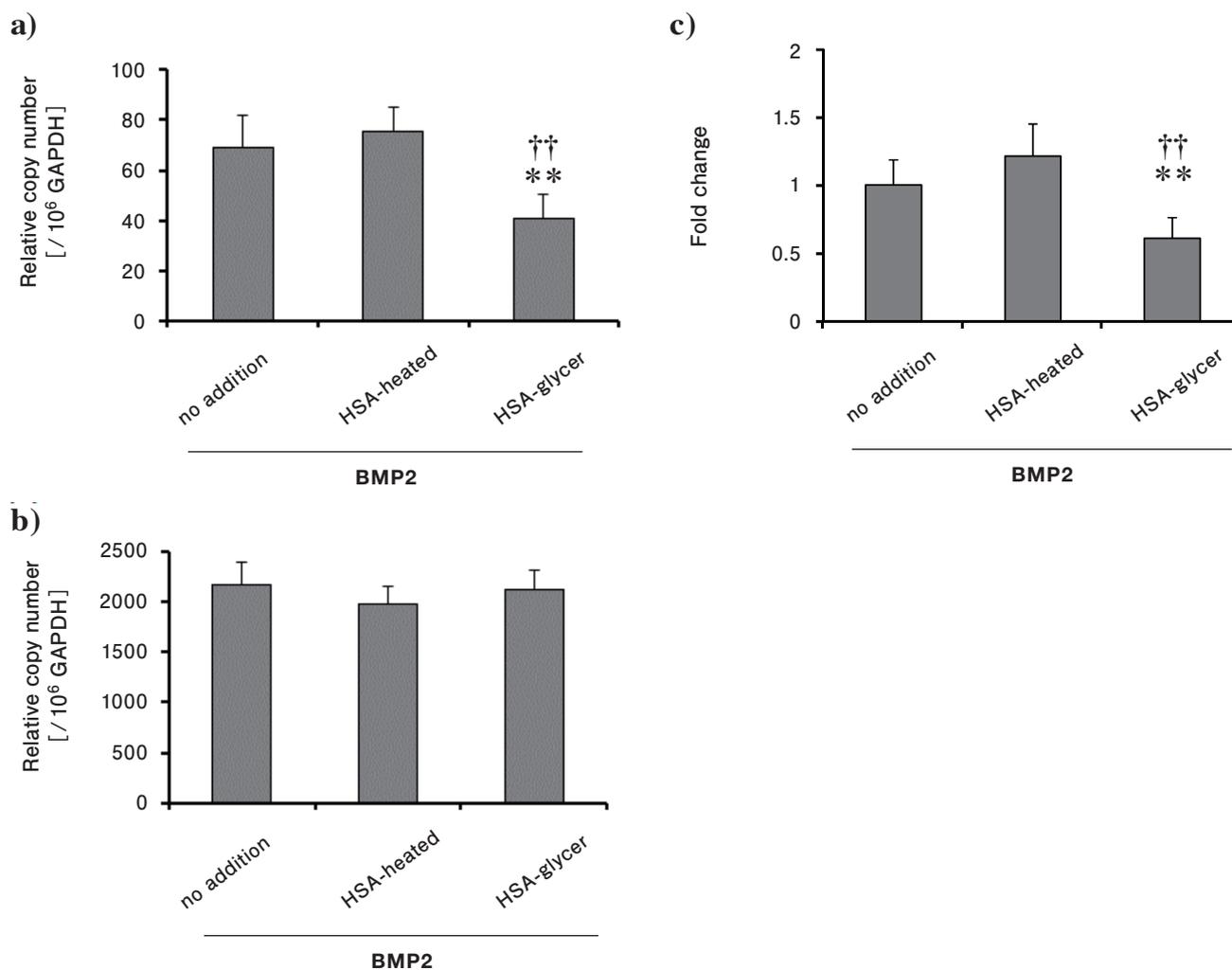

Fig. 5. Glyceraldehyde-derived glycated proteins inhibited BMP2-induced RANKL, but not OPG.

C2C12 cells were treated with 400µg/mL HSA-glycer and 400ng/mL BMP2 for 48 h. qPCR analyses were performed for **(a)** RANKL and **(b)** OPG. **(c)** The ratio of RANKL/OPG. All data obtained were normalized by GAPDH and shown as the mean ± SD (n = 4). ** $p < 0.01$ vs. BMP2. †† $p < 0.01$ vs. BMP2 with heated-HSA. HSA, human serum albumin; BMP2, bone morphogenetic protein 2; RANKL, receptor activator of NF-κB ligand; OPG, osteoprotegerin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SD, standard deviation.

Discussion

Osteoporosis is caused by a decrease in bone strength due to bone mass reduction and deterioration of bone quality, and furthermore leads to fractures and lowers quality of life in the elderly. Since osteoporosis is prevalent in diabetic patients as well as in postmenopausal women, studies have been conducted on the relationship between osteoporosis and glycative stress, a condition in which a hyperglycemic state continues and AGEs are produced in large quantities. For the maintenance of bone quality, the balance between osteoblasts and osteoclasts is important for smooth remodeling. In this study, we elucidated the effects of glycation stress on bone quality using C2C12 cells, focusing on the process of osteoblast differentiation.

C2C12 is an immortalized mouse myoblast cell line, and Katagiri T. *et al.* reported that BMP2 differentiate C2C12 cells to osteoblast cells[32]. As shown in *Fig.1*, BMP2-dependent calcification and expression of ALP and OC, osteoblast markers, was observed in C2C12 cells. Under these conditions, the effects of glycated proteins were verified.

The AGE-related substances used this time are glycelaldehyde[33] generated during the process of fructose metabolism and glycelaldehyde-derived glycated protein (HSA-glycer) generated from HSA, which is the most abundant protein in human blood. As a result, HSA-glycer suppressed BMP2-induced osteoblast differentiation markers and ALP activity (*Fig. 2*).

Other than our study, it has been reported that glyceraldehyde-derived AGEs reacted with bovine serum albumin (BSA) suppresses OC mRNA expression, ALP activity, and calcification in ST2 cells and stromal cells isolated from mouse bone marrow[34]. Also, with regard to glycolaldehyde, Notsu M. *et al.*[35] have reported that AGEs obtained by reacting with BSA suppresses calcification and ST mRNA expression in ST2. These reports indicate that AGEs derived from reaction between serum albumin and glyceraldehyde / glycolaldehyde induce osteoblast differentiation, although the cells used were different from those used in this study.

AGEs derived from glyceraldehyde and glycolaldehyde are also called toxic AGEs and have been reported to exhibit cytotoxicity[36, 37]. In ST2 cells, AGEs were shown to be involved not only in osteoblast differentiation but also in induction of cell death[34, 35], while the glycated protein used in this experiment did not show cytotoxicity (*Fig. 2-a*). This may be mainly due to the different conditions in the glycative reaction. Our results suggest that the effect of cytotoxicity on the suppression of osteoblast differentiation is small.

Runx2 and osterix are known as transcription factors essential for osteoblast differentiation. Runx2 has been reported to be involved in the regulation of OC and collagen type I expression[38]. Osterix is known to be regulated by Runx2[39], and is, in the report of Matsubara T. *et al.*[40], also expressed in mesenchymal cells isolated from Runx2-deficient mice. These findings indicate that the osterix expression mechanism has both Runx2-dependent and -independent pathways.

In this study, we examined the effect of HSA-glycer on Runx2 and osterix expression (*Fig. 3*). As a result, Runx2 expression was weakly inhibited by about 20% due to glyceraldehyde-derived AGEs in both mRNA and protein (*Fig. 3-b,c*). However, osterix mRNA expression was suppressed by more than 50% (*Fig. 3-a*). Therefore, it is possible that AGEs may regulate the induction mechanism of Runx2-independent osterix expression.

Miranda, C. *et al.*[41] also conducted clinical trials in patients with both type 2 diabetes and osteoporosis, in which diabetic patients have been observed to suppress the expression of Runx2 and osterix compared to healthy subjects. This also suggests that AGEs, which are accumulating in diabetic patients, may affect bone metabolism through abnormalities of these transcription factors.

In this study, it was shown that HSA-glycer suppresses osteoblast differentiation by suppressing the phosphorylation of Smad generated through the activation of BMP2 receptors (*Fig. 4*). Regarding glycative stress and Smad signals, so far, Sassi-Gaha, S. *et al.*[42] have reported that AGEs derived from 3-deoxyglucosone (3-DG), an intermediate in glycative reaction, suppress the expression of collagen type I and increase the expression of Smad 7 which suppresses the Smad pathway. However, there are few reports on the effects of glycation stress on Smad expression. For this question, we have shown that, although the level of inhibition of SSA phosphorylation by HSA-glycer was about 30% (*Fig. 4*), the experimental results showed a marked inhibition of osteoblast differentiation (*Fig. 2*). These differences will need further verification in the future.

We have shown that HSA-glycer suppresses osteoblast differentiation, and in order to further evaluate the balance of bone metabolism, the RANKL/OPG ratio, an index of bone metabolism[40, 43, 44], was verified (*Fig. 5*). RANKL is an osteoclast differentiation promoting factor produced from osteocytes and osteoblasts and OPG produced from osteoblasts is an osteoclast differentiation inhibitory factor. Ideally, a constant RANKL/OPG ratio would be required to maintain a balance of bone modeling between osteoblast and osteoclast, however, our study showed that the ratio of RANKL/OPG was reduced by the addition of HSA-glycer (*Fig. 5-c*). It is supposed that, when the value of the RANKL/OPG ratio is low, bone resorption is slowed by inhibiting osteoclast differentiation, thus resulting in the slowing of bone metabolism. Low bone turnover is thought to cause a deterioration in bone quality and a decrease in bone strength. In fact, a decrease in the RANKL/OPG ratio in diabetic patients suffering from osteoporosis has been noted[41, 43].

Finally, this study showed that HSA-glycer suppresses osteoblast differentiation. We have reported that the same HSA-glycer also suppresses RANKL-induced osteoclast differentiation of mouse macrophage-like cells RAW264.7[45]. Coupled with these findings, it is suggested that glyceraldehyde-derived glycated protein inhibits both osteoblast differentiation and osteoclast differentiation and, as a result, promotes low turnover bone metabolism. So far, our laboratory has discovered that more than 500 kinds of plants that have the

effect of inhibiting AGE production. Nowadays, various osteoporosis drugs are on the market. We plan to study the effects of plants that inhibit AGE production from the viewpoint that AGEs are involved in the development of osteoporosis by causing abnormal bone remodeling.

Conclusion

This study demonstrated that glyceraldehyde-derived glycated protein inhibits BMP2-induced osteoblastgenesis via inhibition of Smad pathways. It also might be associated with low bone turnover, leading to low bone quality.

Conflict of Interest Statement

The authors claim no conflict of interest in this study.

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