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Advanced glycation end products suppress osteoblastic differentiation of stromal cells by activating endoplasmic reticulum stress

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ABSTRACT

Advanced glycation end products (AGEs) are involved in bone quality deterioration in diabetes mellitus. We previously showed that AGE2 or AGE3 inhibited osteoblastic differentiation and mineralization of mouse stromal ST2 cells, and also induced apoptosis and decreased cell growth. Although quality management for synthesized proteins in endoplasmic reticulum (ER) is crucial for the maturation of osteoblasts, the effects of AGEs on ER stress in osteoblast lineage are unknown. We thus examined roles of ER stress in AGE2- or AGE3-induced suppression of osteoblastogenesis of ST2 cells. An ER stress inducer, thapsigargin (TG), induced osteoblastic differentiation of ST2 cells by increasing the levels of Osterix, type 1 collagen (Col1), alkaline phosphatase (ALP) and osteocalcin (OCN) mRNA. AGE2 or AGE3 suppressed the levels of ER stress sensors such as IRE1α, ATF6 and OASIS, while they increased the levels of PERK and its downstream molecules, ATF4. A reduction in PERK level by siRNA did not affect the AGEs-induced suppression of the levels of Osterix, Col1 and OCN mRNA. In conclusion, AGEs inhibited the osteoblastic differentiation of stromal cells by suppressing ER stress sensors and accumulating abnormal proteins in the cells. This process might accelerate AGEs-induced suppression of bone formation found in diabetes mellitus.

1. Introduction

Both osteoporosis and diabetes mellitus (DM) are major diseases among elderly people, and a plenty of investigations have been performed on the relationships between osteoporotic fractures and DM. Recent studies have shown that hip and spine fracture risks are increased in patients with type 1 (T1) or type 2 (T2) DM. Patients with T1DM have decreased bone mineral density (BMD) and a 6.9-fold higher risk of hip fracture than non-DM controls, while those with T2DM have a 1.4-fold higher risk of hip fracture despite normal or even increased BMD [1]. The presence of T2DM is also a risk factor for prevalent vertebral fractures (VFs) with odds ratios of 1.86 in women and 4.73 in men [2]. These findings suggest that bone fragility, which is not defined by BMD, relates to increased fracture risks in T2DM patients, and that impaired bone quality is probably involved in this process.

Non-enzymatic reactions of carbohydrates as well as oxidized lipid with proteins induce the production of advanced glycation end products (AGEs) [3,4]. The accumulation of AGEs is a characteristic feature of the tissues in aged people and DM patients. Previous studies showed that AGEs, especially AGE2 and AGE3, are related to DM complications [5,6]. It is also documented that AGEs adversely affect bone. In vitro, we showed that the combination of high glucose and AGE2 or AGE3 inhibited the mineralization of osteoblastic MC3T3-E1 cells through glucose-induced increase in RAGE expression [7]. Franke et al. showed that AGE-modified bovine serum albumin (AGE-BSA) suppressed cell number and osteogenic markers such as type 1 collagen (Col1), osteocalcin (OCN), and alkaline phosphatase (ALP) in human osteoblasts [8]. They also found that AGE-BSA induced osteoclastogenic properties of osteoblasts such as RANKL and osteoprotegerin. Moreover, we reported that AGE2 and AGE3 inhibited the osteoblastic differentiation and mineralization of mouse stromal ST2 cells by decreasing Osterix expression and increasing RAGE expression [9]. However, it remains unclear what mechanisms are involved in these detrimental effects of AGEs on bone cells and fractures.

The ER is crucial for biosynthesis, folding and modification of proteins [10]. Increased unfolded proteins in ER causes ER stress, and they are eliminated through its sensors. It is known that inositol-requiring transmembrane kinase and endonuclease (IRE) 1α as

Abbreviations: ATF, activating transcription factor; IRE, inositol-requiring transmembrane kinase and endonuclease; OASIS, old astrocyte specifically induced substance; PERK, protein kinase RNA-like endoplasmic reticulum kinase.

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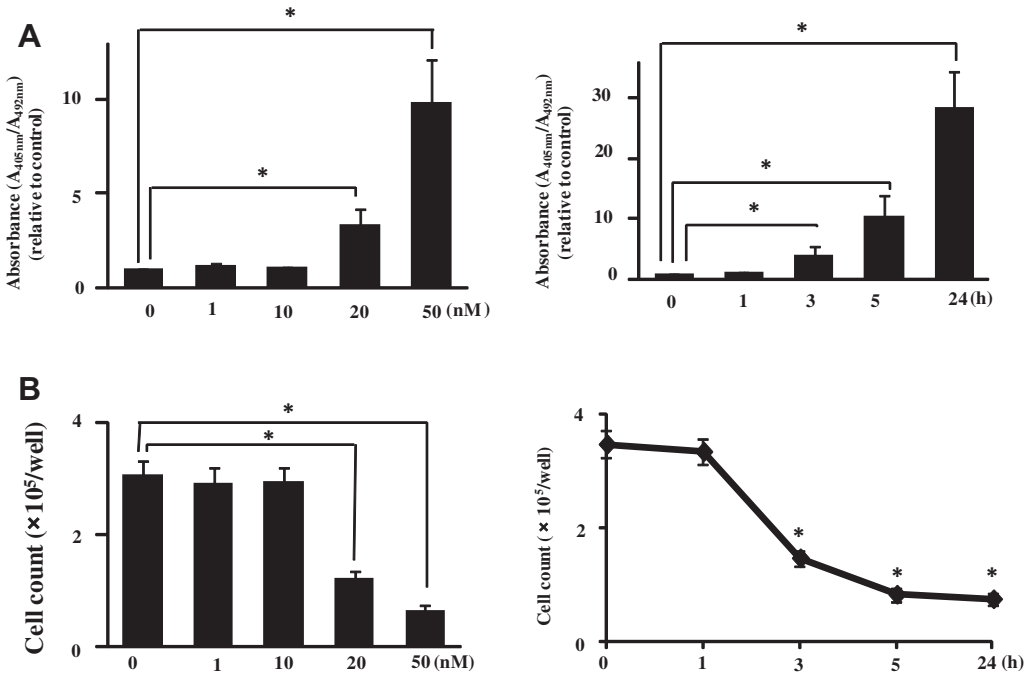

Fig. 1. Effects of TG on the apoptosis and cell number of ST2 cells. (A) The cells were seeded on 96-well plates at a density of 2×10^4 cells/well and were incubated overnight. On the next day, the cells were treated with 0–50 nM TG for 1 h, or with 10 nM TG for 0–24 h. The apoptotic cell death was analyzed by an ELISA for DNA fragments by an absorbance at 405 nm. $^*P < 0.01$ compared to control. The results were the representative of 3 separate experiments, and were expressed as the mean ± S.E. over control values ($n = 6$). (B) The cells were seeded on 6-well plates at a density of 3000 cells/well, and were incubated overnight. On the next day, the cells were treated with 0–50 nM TG for 1 h, or with 10 nM TG for 0–24 h. The cell counting was performed by a hemocytometer after Trypan blue exclusion.$^*P < 0.01$. The results were the representative of 3 separate experiments, and were expressed as the mean ± S.E. over control values ($n = 6$).

well as activating transcription factor (ATF) 6 act as ER stress sensors and participate in unfolded protein response, which transmit the information of increased unfolded proteins to the nucleus, and results in increased transcription of target genes such as X-box binding protein (XBP)1 [11–14]. Saito et al have recently found a novel ER stress sensor, old astrocyte specifically induced substance (OASIS) [15]. OASIS is a basic leucine zipper transcription factor localized in the ER membrane. ER stress enhances the cleavage of N-terminal cytoplasmic domain of OASIS, which translocates into nucleus and activates the transcription of target genes [16]. Osteoblasts are known to synthesize a large amount of proteins and to secrete them into the bone matrix during their differentiation. Thus, it is crucial for osteoblasts to produce good quality bone matrix proteins by removing unfolded ones through ER stress response to complete their maturation in microenvironments. Murakami et al. revealed that OASIS induced bone formation through the expression of Col1 and the secretion of bone matrix proteins [17], suggesting the beneficial effect of ER stress on bone. However, it is unknown whether or not AGEs modulate ER stress in osteoblasts and affect their differentiation.

In this study, we therefore examined the effects of AGE2 and AGE3 on ER stress sensors such as IRE1α, ATF6, PERK and OASIS in ST2 cells to clarify relationships between ER stress and AGEs-induced suppression of osteoblastogenesis in the cells.

2. Materials and methods

2.1. Materials

Human (h) recombinant BMP-2 was kindly provided by Astellas Pharmaceutical Co., Ltd. (Tokyo, Japan). Thapsigargin was obtained from Wako Pure Chemical Industries (Tokyo, Japan). An anti-β-actin antibody was obtained from Sigma–Aldrich Corp. (St. Louis, MO). Anti-ALP, anti-IRE1α, anti-phospho-PERK, anti-PERK, anti-ATF4 antibodies, PERK small interfering (si) RNA and control siRNA were from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody for phospho-IRE1α was from abcam (Tokyo, Japan). An antibody for Col1 was from Calbiochem and Ingenex Corp. (San Diego, CA). All other chemicals used were of analytical grade.

2.2. Cell culture

Mouse ST2 cells were cultured in α-minimum essential medium (α-MEM) containing 5.5 mmol/L glucose. This medium was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin (Gibco-BRL) in 5% CO_2 at 37 centigrade and changed twice a week. Cells were treated with various agents after reaching confluence except for apoptosis and cell growth experiments. For induction of osteoblastic differentiation, the cells were cultured in α-MEM supplemented with 100 ng/mL BMP-2 plus 10% FBS, 1% penicillin–streptomycin, 5 mM -glycerophosphate and 100 μg/mL ascorbic acid, after reaching confluence.

2.3. Preparation of AGEs

AGE-BSA was prepared as described previously [7]. AGE2 and AGE3 were prepared by incubating 50 mg/mL BSA (Sigma, St. Louis, MO) with 0.1 M DL-glyceraldehyde (Nacalai Tesque, Kyoto, Japan) and 0.1 M glycolaldehyde (Sigma), respectively, under sterile conditions in 0.2 M phosphate buffer (pH 7.4) containing 5 mM diethylene-triamine-pentaacetic acid (DTPA) at 37 centigrade for 7 days. Nonglycated BSA was incubated under the same conditions except for the absence of DL-glyceraldehyde or glycolaldehyde as a negative control. Then low molecular weight reactants and aldehydes were removed using a PD-10 column chromatography and dialysis against phosphate-buffered saline (PBS).

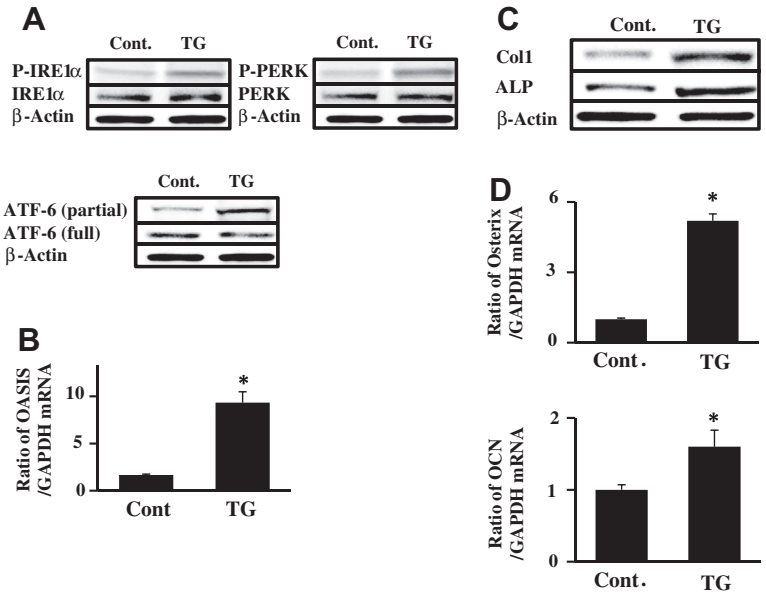

Fig. 2. Effects of TG on osteoblastic differentiation of ST2 cells. (A and C)After reaching confluence, ST2 cells were treated with or without 10 nM TG for 1 h every day, and the medium was replaced with fresh α-MEM containing 100 ng/mL BMP-2 after treatments. Total protein was extracted from the cells on day 7 and Western blot analyses for indicated proteins were performed. (B and D) After reaching confluence, ST2 cells were treated with or without 10 nM TG for 1 h every day, and the medium was replaced with fresh α-MEM containing 100 ng/mL BMP-2 after treatments. Total RNA was extracted on day 7, and real-time PCR for OASIS, Osterix, OCN, or GAPDH was performed. Data are expressed relative to the GAPDH mRNA value. $^{*}P < 0.01$.

2.4. Protein extraction and Western blot analysis

Cells were lysed with radioimmunoprecipitation buffer containing 0.5 mM phenylmethylsulfonylfluoride, complete protease inhibitor mixture (Roche Applied Science, Tokyo, Japan), 1% Triton X-100 and 1 mM sodium orthovanadate. Proteins were transferred in 25 mM Tris, 192 mM glycine and 20% methanol to polyvinylidene difluoride. Blots were blocked with 20 mM Tris–HCl (pH 7.5), 137 mM NaCl, 0.1% Tween 20 and 3% dried milk powder. The membranes were immunoblotted with each primary antibody. The antigen–antibody complexes were visualized using the appropriate secondary antibodies (Sigma–Aldrich Corp.) and an enhanced chemiluminescence detection system, LAS-4000 IR multi color (FUJIFILM). The results depicted in each figure are representative of at least three independent cell preparations. Each experiment was repeated three times.

2.5. RNA extraction and real-time PCR

Total RNA was prepared from cells using Trizol reagent (Invitrogen, San Diego, CA). cDNA was synthesized using a SuperScript-III cDNA synthesis kit (Invitrogen). Specific mRNA was quantified by using an ABI PRISM 7000 sequence detection system (Applied Biosystems Inc.) with SYBR Premix Ex TaqTMII (Perfect Real Time) kits (TaKaRa) according to the manufacturer's standard protocol. The mRNA value for each gene was normalized relative to the mouse GAPDH mRNA levels in RNA samples. Primer sequences (forward and reverse) were as follows:

GAPDH, 5′-GTGTACATGGTTCCAGTATGAGTCC-3′ and 5′-AGTGAGTTGTCATATTTCTCGTGGT-3′; OCN, 5′-CCTGAGTCTGACAAAGCCTTCA-3′ and 5′-GCCGGAGTCTGTTCACTACCTT-3′; Osterix, 5′-AGCGACCACTTGAGCAAACAT-3′ and 5′-GCGGCT GATTGGCTTCTTCT-3′; Col1, 5′-ATGCCTGGTGAACGTGGT-3′ and 5′-AGGAGAGCCATCAGCACCT-3′; OASIS, 5′-CCTTGTGCCTGTCAAGATGGAG-3′ and 5′-GCA GCAGCCATGGCAGAGGAG-3′.

2.6. siRNA transfection

Mouse PERK siRNA and control siRNA were transfected into ST2 cells with LipofectAMINE (Invitorgen). Six hours later, the cells were fed with fresh medium containing 10% FBS, and the transfected cells were harvested for 48 h and were used for the experiments.

2.7. Evaluation of cell growth

Cell viability was evaluated by cell count. ST2 cells were seeded on 6-well plates at a density of 3000 cells/well, and were incubated overnight in α-MEM with 10% FBS. On the next day, the cells were treated with 0–50 nM thapsigargin for 1 h, or with 10 nM thapsigargin for 0–24 h. The cell counting was performed by a hemocytometer after Trypan blue exclusion.

2.8. Measurement of apoptotic cell death

ST2 cells were seeded on 96-well plates at a density of 2×10^4 cells/well and were incubated overnight in α-MEM with 10% FBS and antibiotics. On the next day, the cells were treated with 0–50 nM thapsigargin for 1 h, or with 10 nM thapsigargin for 0–24 h. The apoptotic cell death was analyzed in an enzyme-linked immunosorbent assay (ELISA) for DNA fragments (Cell Death Detection ELISA Plus, Roche Molecular Biochemicals).

2.9. Statistics

All experiments were repeated at least three times. Data are expressed as mean ± S.E. Statistical analysis was performed using analysis of variance. A P value <0.05 was taken to indicate a significant difference.

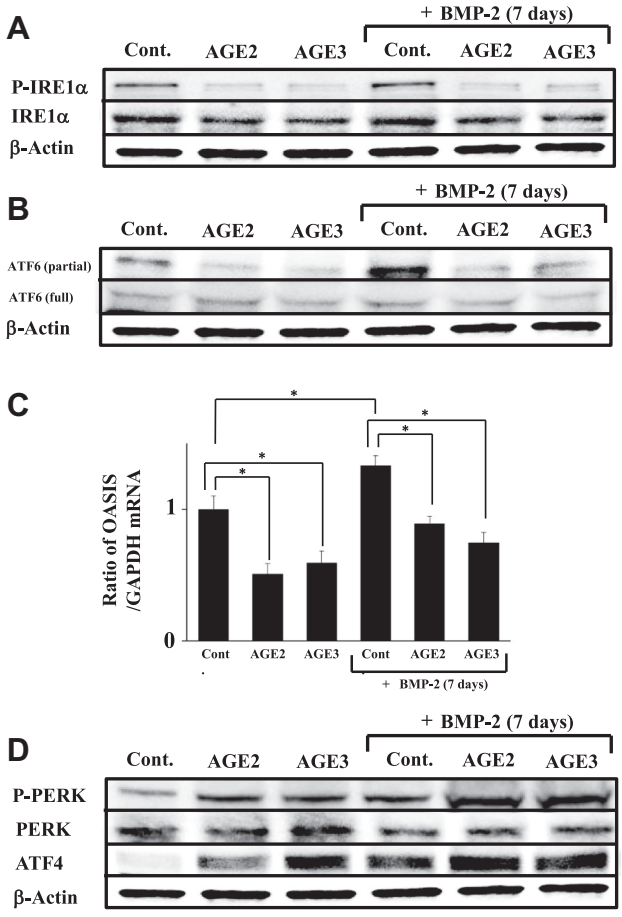

Fig. 3. Effects of AGE2 or AGE3 on the expressions of ER stress sensors in ST2 cells. (A, B and D) After reaching confluence, ST2 cells were treated with 200 μg/mL each of control BSA, AGE2 or AGE3 in the presence or absence of 100 ng/mL BMP-2 for 7 days. Total protein was extracted, and Western blot analyses for indicated proteins were performed. (C) After reaching confluence, ST2 cells were treated with 200 μg/mL each of control BSA, AGE2, or AGE3 in the presence or absence of 100 ng/mL BMP-2 for 7 days. Total RNA was extracted, and real-time PCR for OASIS or GAPDH was performed. Data are expressed relative to the GAPDH mRNA value. $^{*}P < 0.01$.

3. Results

3.1. Effects of thapsigargin (TG) on the apoptosis and cell number of ST2 cells

We examined the effects of an ER stress inducer, thapsigargin (TG), on apoptotic cell death or cell number of ST2 cells using an ELISA for DNA fragments or cell counting. As shown in Fig. 1A and B, although administration of 0–10 nM TG did not affect cell mortality and cell number, 20–50 nM TG significantly increased and decreased them, respectively, ($p < 0.01$). In addition, unlike application of 10 nM TG for 0–1 h, incubation with 10 nM TG for 3–5 h significantly increased and decreased cell mortality and cell number, respectively, ($p < 0.01$). Thus, we treated the cells at 10 nM TG for 1 h in the following experiments to avoid excessive ER stress that induces apoptosis and decreases cell number.

3.2. Effects of TG on osteoblastic differentiation of ST2 cells

Next, we examined the effects of TG on the expressions of ER stress sensors such as IRE1α, PERK, ATF6 and OASIS. TG increased the levels of phospho-IRE1α, phospho-PERK and activated ATF6 (partial) proteins in ST2 cellsby Western blot analyses (Fig. 2A). Moreover, TG significantly enhanced the levels of OASIS mRNA in ST2 cells by real-time PCR ($p < 0.01$) (Fig. 2B).

In addition, we examined the effects of TG on the osteoblastic differentiation of ST2 cells. TG increased the levels of Col1 and ALP proteins as well as those of Osterix and OCN mRNA in the cells by Western blot analyses (Fig. 2C) and real-time PCR (Fig. 2D), respectively, and the latter findings were significant ($p < 0.01$).

3.3. Effects of AGE2 or AGE3 on the expressions of ER stress sensors in ST2 cells

We examined the effects of AGE2 or AGE3 on the expressions of ER stress sensors such as IRE1α, PERK, ATF6 and OASIS in ST2 cells. As shown in Fig. 3A and B, treatments of the cells with 200 μg/mL of AGE2 or AGE3 for 7 days suppressed the levels of phospho-IRE1α and activated ATF6 (partial) proteins in the presence or absence of BMP-2 by Western blot analyses. Moreover, the treatments significantly inhibited the level of OASIS mRNA by real-time PCR ($p < 0.01$) (Fig. 3C). In contrast, the treatments increased the levels of phospo-PERK and its downstream molecules, ATF4 proteins in

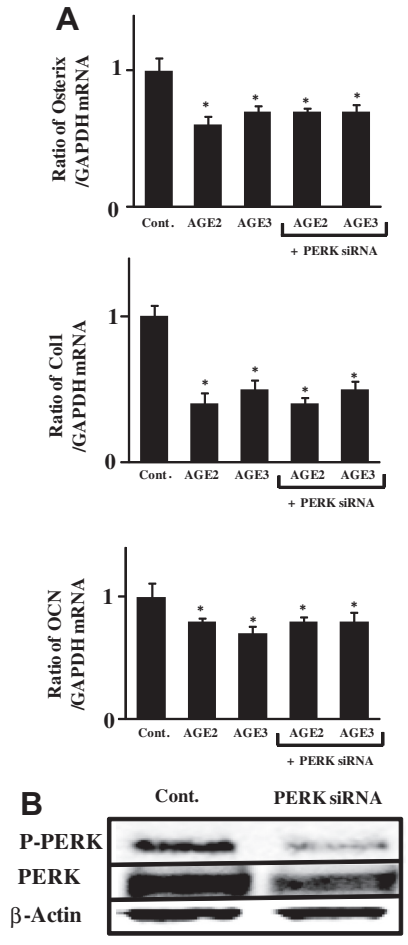

Fig. 4. Effects of PERK reduction by siRNA on the AGEs-induced suppression of osteoblastogenesis of ST2 cells. (A) After reaching confluence, ST2 cells were transfected by control siRNA or PERK siRNA. After transfection, 200 μg/mL AGE2 or AGE3 were added in α-MEM medium containing 100 ng/mL BMP-2. Total RNA was extracted on day 7, and real-time PCR for Osterix, Col1, OCN or GAPDH was performed. Data are expressed relative to the GAPDH mRNA value. $^{*}P < 0.01$. (B) After reaching confluence, ST2 cells were transfected by control siRNA or PERK siRNA. After transfection, 200 μg/mL AGE2 or AGE3 were added in α-MEM medium containing 100 ng/mL BMP-2. Total protein was extracted on day 7, and Western blot analyses for anti-phospho-PERK, PERK and β-actin were performed.

the presence or absence of BMP-2 by Western blot analyses (Fig. 3D).

3.4. Effects of the reduction in PERK expression by its siRNA on the AGEs-induced suppression of osteoblastic differentiation of ST2 cells

Next, we examined whether or not the reduction of PERK expression by siRNA would affect the AGEs-induced suppression of osteoblastic differentiation of ST2 cells. As shown in Fig. 4A, although treatments of the cells with 200 μg/mL of AGE2 or AGE3 for 7 days in the presence of BMP-2 significantly inhibited the levels of Osterix, Col1 and OCN mRNA by real-time PCR ($p < 0.01$), the reduction of PERK expression by its siRNA did not affect the extent of these mRNA inhibitions. We confirmed that the levels of both phospho-PERK and PERK proteins were suppressed by PERK siRNA transfection by Western blot analysis (Fig. 4B).

4. Discussion

In the present study, we showed that treatments of mouse stromal ST2 cells with 10 nM TG for 1 h induced the expressions of ER stress sensors such as PERK, IRE1α, ATF6 and OASIS. Moreover, it enhanced osteoblastic differentiation of the stromal cells by increasing the levels of Osterix, Col1, and OCN (Fig. 2). In contrast, TG at higher concentrations and longer treatments induced the apoptosis and decreased the number of the cells (Fig. 1). These findings suggest that an adequate ER stress response, but not excessive one, enhances osteoblastic differentiation of stromal cells by activating ER stress sensors, removing abnormal unfolded proteins, and possibly producing good quality bone matrix proteins.

In this study, we hypothesized that AGEs might suppress osteoblastic differentiation of stromal cells through the interaction with ER stress response pathways involving ER stress sensors, and found that AGE2 or AGE3 suppressed the levels of ER stress sensors such as IRE1α, ATF6 and OASIS (Fig. 3). These ER stress sensors as well as PERK are transmembrane proteins, which sense the accumulation of the unfolded proteins in ER lumen and deliver signal to the cytosol and nucleus, resulting in increasing ER-molecular chaperons to regulate the unfolded proteins. It is documented that ER stress-induced IRE1α increases its autophosphorylation and that phosphorylated IRE1α enhances the splicing of X-box binding protein 1, which induces the transcription of enzymes involving ER-molecular chaperons [18]. On the other hand, ATF6 is delivered into the Golgi apparatus and cleaved by proteases upon the accumulation of ER stress. The N-terminal cytosolic fragment of ATF6 moves into nucleus to activate UPR target genes involving ER-molecular chaperons [19,20].

In contrast, we found that AGE2 or AGE3 increased the levels of PERK and its downstream molecules, ATF4 (Fig. 3). However, a reduction of endogenous PERK did not affect AGEs-induced suppression ofosteoblastic differentiation of stromal cells (Fig. 4), suggesting that the PERK pathway is not involved in the inhibitory effects of AGEs on stromal cell differentiation. These findings indicate that AGE2 or AGE3 increase ER stress excessively by suppressing the levels of ER stress sensors such as OASIS, IRE1α and ATF6 and accumulating unfolded proteins in ER, and suggest that agents adequately stimulating ER stress sensors could be candidates for therapeutic drugs for bone fragility in DM by removing abnormal unfolded proteins accumulated in ER by AGEs and thereby producing good quality bone matrix proteins.

This study has some limitations. First, we used ST2 cells as a model for stromal cells, which are clonal stromal cells isolated from bone marrow of BC8 mice [21]. ST2 cells might not be identical to natural stromal cells in vivo, and we need to use primary bone marrow stromal cells in future. Second, we only examined the level of OASIS mRNA by real-time PCR, but not OASIS protein, because an antibody for OASIS is not commercially available.

In conclusion, we found that AGE2 or AGE3 inhibited the differentiation of stromal cells into osteoblasts by suppressing ER stress sensors such as IRE1α, ATF6 and OASIS. This process seems to be involved in AGEs-induced suppression of osteoblastic differentiation of stromal cells. These mechanisms might partly explain reduced bone formation and enhanced bone fragility in DM patients.

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