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Full title

Advanced Glycation End Product 3 (AGE3) Increases Apoptosis and the Expression of Sclerostin by Stimulating TGF-β Expression and Secretion in Osteocyte-Like MLO-Y4-A2 Cells

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Abstract

Background: Advanced glycation end products (AGEs) cause bone fragility due to deterioration in bone quality. We previously reported that AGE3 induced apoptosis and inhibited differentiation via increased transforming growth factor (TGF)-β signaling in osteoblastic cells. Additionally, we demonstrated that AGE3 increased apoptosis and sclerostin expression and decreased receptor activator of nuclear factor-κB ligand (RANKL) expression in osteocyte-like cells. However, it remains unclear whether TGF-β signaling is involved in the effects of AGEs on apoptosis and the expression of sclerostin and RANKL in osteocytes.

Methods: Effects of AGE3 on apoptosis of mouse osteocyte-like MLO-Y4-A2 cells were examined by DNA fragmentation ELISA. Expression of TGF-β, sclerostin and RANKL was evaluated using real-time PCR, Western blotting, and ELISA kits. To block TGF-β signaling, we used SD208, a TGF-β type I receptor kinase inhibitor.

Results: AGE3 (200 μg/mL) significantly increased apoptosis and mRNA expression of *Sost*, the gene encoding sclerostin, and decreased *Rankl* mRNA expression in MLO-Y4-A2 cells. AGE3 significantly increased the expression of TGF-β. Co-incubation of SD208 with AGE3 significantly rescued AGE3-induced apoptosis in a dose-dependent manner. Moreover, SD208 restored AGE3-increased mRNA and protein expression of sclerostin. In contrast, SD208 did not affect AGE3-decreased mRNA and protein expression of RANKL.

Conclusions: These findings suggest that AGE3 increases apoptosis and sclerostin expression through increasing TGF-β expression in osteocytes, and that AGE3 decreases RANKL expression independently of TGF-β signaling.

Key words

Advanced Glycation End Products, TGF-β, Osteocyte, Bone quality, diabetes mellitus

58 **Abbreviations**

59 DM: diabetes mellitus, BMD: bone mineral density, AGEs: Advanced glycation end products, TGF-β:
60 transforming growth factor-β, RANKL: nuclear factor-κB ligand, BSA: bovine serum albumin, α-MEM: α-
61 minimum essential medium

62

Introduction

Osteoporosis and diabetes mellitus (DM) are increasing with population aging and have become important problems worldwide because both diseases affect quality of life and increase morbidity and mortality [1-4]. According to World Health Organization osteoporosis categorization, 2.5 standard deviations below young adult mean bone mineral density (BMD) are defined as osteoporosis[5]. However, not only BMD reduction but also deterioration of bone quality is considered to be an important component of bone fragility, because osteoporotic fracture is often occurred in patients with normal BMD. Previous studies have shown that patients with type 2 DM (T2DM) have a 1.4- to 4.7-fold increased risk of fractures although they have normal or slightly higher than normal BMD [6-8]. Thus, osteoporosis is recently recognized as one of the diabetic complications. Although underlying mechanisms of diabetes-related bone fragility remains unclear, it is suggested that the increased risk of fractures is mainly due to the deterioration of bone quality in patients with T2DM.

Previous studies showed that higher serum or urine pentosidine levels were significantly associated with the risk of fractures in patients with T2DM [9, 10]. Advanced glycation end products (AGEs) are produced via nonenzymatic chemical modification of proteins under conditions of hyperglycemia and oxidative stress. Particularly in patients with T2DM, formation of AGEs is increased, and AGEs are reported to cause bone fragility through deterioration of collagen [11], inhibition of osteoblastic differentiation [12, 13], and deterioration of micro-architecture [14]. There are several types of AGE, and AGE3 was produced by glycolaldehyde. We previously showed that AGE2 and AGE3 suppressed cell growth, increased apoptosis of osteoblastic cells [12], and inhibited the differentiation and mineralization of the cells [12, 13]. Because previous studies have shown that AGE3-TGF-β signaling is involved in diabetic complications [15, 16], we have focused on the effects of AGE3 on bone cells through transforming growth factor (TGF)-β signaling. Indeed, our recent study revealed that AGE3 suppressed mineralization by increasing the expression and secretion of TGF-β in cells of the osteoblast lineage

[17].

Osteocytes are the most abundant cells in bone and are recognized as central regulators of bone homeostasis. They are an important source of sclerostin, the main inhibitor of osteoblast activity, and of RANKL, the most important regulator of osteoclastogenesis. However, little is known about the association between bone fragility and the osteocyte network in T2DM. Recently, we showed for the first time that AGE3 increased apoptosis and the expression of sclerostin, and decreased the expression of receptor activator of nuclear factor-κB ligand (RANKL) in osteocyte-like MLO-Y4-A2 cells [18]. However, the mechanisms by which AGE3 affects apoptosis and the expression of sclerostin and RANKL are still unknown.

AGEs cause diabetic microvascular complications such as nephropathy and neuropathy via activating TGF-β signaling. For example, AGEs increased TGF-β expression and activated Smad signals in mesangial and nerve cells [19, 20]. On the other hand, it has been shown that TGF-β is expressed in osteocytes [21]. Loots *et al.* demonstrated that TGF-β increased the expression of *Sost*, the gene encoding sclerostin, in UMR106.01, a rat osteosarcoma cell line [22]. However, because the roles of TGF-β in osteocytes are still unclear, we hypothesized that AGEs may affect osteocytes via TGF-β signaling. Therefore, in this study, we aimed to examine whether TGF-β signaling is involved in AGE3-induced apoptosis and altered expression of sclerostin and RANKL in osteocytic MLO-Y4-A2 cells.

Materials and methods

Materials

Cell culture medium and supplements were purchased from GIBCO-BRL (Rockville, MD). SD208, a TGF-β type I receptor kinase inhibitor, was purchased from TOCRIS (Bristol, United Kingdom). Anti-β-actin antibody was obtained from Sigma (St. Louis, MO, USA). Anti-sclerostin antibody was purchased from Abcam (Tokyo, Japan). Anti-RANKL antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bovine serum albumin (BSA), glycolaldehyde, and mouse monoclonal antibodies were purchased from Sigma. All other chemicals were of the highest grade available commercially.

Cell culture

As previously described [18], we use MLO-Y4-A2, a murine long bone-derived osteocytic cell line, which was kindly provided by Dr. Y. Kato (Asahi Kasei Medical Corporation, Tokyo, Japan) and Dr. Lynda F. Bonewald (University of Missouri). Briefly, the cells were cultured on type I collagen-coated plates in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin in 5% CO_2 at 37 °C. The culture medium was changed twice weekly.

Preparation of AGE3

AGE3 was obtained as described previously [13]. Briefly, AGE3 was prepared by incubating 50 mg/mL BSA with 0.1 M glycolaldehyde at 37 °C for 7 days under sterile conditions in 0.2 M sodium phosphate buffer (pH 7.4) containing 5 mM diethylenetriamine-pentaacetic acid. As a negative control, nonglycated BSA was incubated under the same conditions, except for the absence of glycolaldehyde. After the incubation period, low-molecular weight reactants and aldehydes were removed using a PD-10 column (GE Healthcare Bio-

124 Sciences AB, Uppsala, Sweden) and dialyzing against phosphate-buffered saline (PBS).

125

126 *Quantification of TGF-β1 and RANKL proteins*

127 The concentration of TGF-β1 in whole-cell lysates was determined by using commercially available
128 diagnostic kits (R&D Systems, Minneapolis, MN). In brief, samples were activated with 1 N HCl for 10 min at
129 room temperature, followed by neutralization with 1.2 N NaOH in HEPES. Samples were added to the wells of
130 microtiter plates coated with a monoclonal anti-TGF-β1 antibody. After incubation of the sample for 2 h at room
131 temperature, a horseradish peroxidase-conjugated polyclonal antibody against TGF-β1 was added and the plates
132 were incubated for an additional 2 h. The assay was developed with peroxidase substrate for 30 min at room
133 temperature in the dark. Absorbance was measured at 450 nm, and a reference wavelength of 540 nm was used.
134 Standard curves for TGF-β1 were prepared using serial dilutions of purified TGF-β1.

135 As previously described [18], the concentration of RANKL in whole-cell lysates was determined by
136 using commercially available diagnostic ELISA kits (Abcam Corp., Tokyo, Japan).

137

138 *Quantification of gene expression by real-time PCR*

139 SYBR Green chemistry in conjunction with real-time PCR was used to quantify the amounts of
140 mRNAs for *Tgf-β*, *Rage*, *Sost*, *Rankl*, *Opg* and *36B4*, a housekeeping gene, according to an optimized protocol
141 [13, 23, 24]. Total RNA was isolated using Trizol reagent (Invitrogen, San Diego, CA) and further purified by
142 two successive phenol-chloroform extractions. First-strand cDNA was synthesized with an oligo-dT primer and
143 a SuperScript-III cDNA synthesis kit (Invitrogen). Sense and antisense oligonucleotide primers were designed
144 according to published cDNA sequences using Primer Express (version 2.0.0, Applied Biosystems, Carlsbad,
145 CA). The cDNA was amplified with an ABI PRISM 7000 sequence detection system (Applied Biosystems Inc.).

146 The cDNA-specific SYBR Green Mix was incorporated into the PCR buffer provided in the QuantiTect SYBR
147 PCR kit to allow for quantitative detection of the PCR product in a 25-μL reaction volume. The temperature
148 profile of the reaction was 60 °C for 2 min, followed by 95 °C for 15 min and 40 cycles of denaturation at 94 °C
149 for 15 s and annealing and extension at 60 °C for 1 min. Primer sequences were as follows: *Tgf-β*, 5′-
150 TGAACCAAGGAGACGGAATACAGG-3′ and 5′-GCCATGAGGAGCAGGAAGGG-3′; *Rage*, 5′-
151 ACAACCAACTCGGACTTCCA-3′ and 5′-GTCGTTTTCGCCACAGGATA-3′; *Sost*, 5′-
152 TCCTCCTGAGAACAACCAGAC-3′ and 5′-TGTCAGGAAGCGGGTGTAGTG-3′; *Rankl*, 5′-
153 TGCTTGTGAC-GAGCTATCAG-3′ and 5′-GAGGACAGGGAGGATCAAGT-3′; Opg, 5′-
154 AGCTGCTGAAGCTGTGGAA-3′ and 5′-TGTTCGAGTGGCCGAGAT-3′; *36B4*, 5′-
155 AAGCGCGTCCTGGCATTGTCT-3′and 5′-CCGCAGGGGCAGCAGTGGT-3′.
156
157
158 *Western blotting*
159 Cells were lysed with radioimmunoprecipitation buffer containing 0.5 mM
160 phenylmethylsulfonylfluoride, complete protease inhibitor mixture (Roche Applied Science, Tokyo, Japan), 1%
161 Triton X-100, and 1 mM sodium orthovanadate. Proteins were transferred in 25 mM Tris, 192 mM glycine, and
162 20% methanol to polyvinylidene difluoride membrane. Blots were blocked with 20 mM Tris HCl (pH 7.5), 137
163 mM NaCl, 0.1% Tween-20, and 3% dried milk powder. The membranes were immunoblotted with each primary
164 antibody. The antigen-antibody complexes were visualized by using the appropriate secondary antibodies and an
165 enhanced chemiluminescence detection system (LAS-4000 IR multi-color imager, FUJIFILM, Tokyo, Japan).
166 National Institutes of Health (NIH) image software (ImageJ) was used to quantify the area of band. The average
167 value of band in six randomly selected fields was used to evaluate the protein expressions of Sclerostin and Rankl.

The results depicted in each figure are representative of at least three independent cell preparations.

Assessment of apoptotic cell death

Apoptosis was assessed by using Cell Death Detection ELISA Plus kit (Roche Molecular Biochemicals, IN, USA) according to the manufacturer's protocol. MLO-Y4-A2 cells were seeded in 96-well plates at a density of 3000 cells/well and incubated overnight at 37 °C in α-MEM. The next day, the cells were treated with either BSA (negative control) or AGE3. On day 5 after treatment, the cells were lysed in 200 μL lysis buffer. After centrifugation, 20 μL of the supernatant was transferred to a streptavidin-coated microplate and exposed to anti-histone antibody (biotin-labeled) and anti-DNA antibody (peroxidase-conjugated) for 2 h at room temperature. Each well was washed 3 times with the incubation buffer, and antibody-nucleosome complexes bound to the microplate were determined spectrophotometrically using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). The absorbance measured at 405 nm was proportional to the degree of apoptosis. The results were expressed relative to the control.

Statistical analysis

Results are expressed as mean ± SEM. Differences between groups were evaluated by one-way analysis of variance (ANOVA), followed by Fisher's protected least significant difference test. For all statistical tests, a value of $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of AGE3 on apoptosis and expression of Sost and Rankl mRNA in MLO-Y4-A2 cells

To confirm our previous findings [18], we examined the effects of AGE3 on apoptosis and the expression of *Sost* and *Rankl*. As shown in Fig. 1, incubation with 200 μg/mL AGE3 significantly increased apoptosis ($p < 0.001$) (Fig. 1A) and the expression of *Sost* mRNA ($p < 0.01$) (Fig. 1B), and significantly decreased *Rankl* mRNA ($p < 0.01$) (Fig. 1C).

Effects of AGE3 on TGF-β as well as Rage expression in MLO-Y4-A2 cells

We examined next the effects of AGE3 on the expression of TGF-β mRNA and protein. Quantitative real-time PCR showed that 200 μg/mL AGE3 significantly increased the expression of TGF-β mRNA at 12 h and 24 h after treatment ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 2A). Moreover, the TGF-β ELISA assay showed that 200 μg/mL AGE3 significantly increased TGF-β protein levels on days 3 and 5 after treatment ($p < 0.001$ and $p < 0.001$, respectively) (Fig. 2B). Real-time PCR also showed that AGE3 significantly increased the expression of Rage, which is a receptor for AGEs, on MLO-Y4-A2 cells ($p < 0.01$) (Fig. 2C).

Involvement of TGF-β signaling in the AGE3-induced apoptosis of MLO-Y4-A2 cells

We examined whether or not TGF-β signaling is involved in the effect of AGE on apoptosis by using SD208, a TGF-β type I receptor kinase inhibitor. The DNA fragmentation ELISA showed that 200 μg/mL AGE3 significantly increased the apoptosis ($p < 0.001$), and that the apoptotic effect of AGE3 was significantly decreased by co-incubation with SD208 (1.0 and 2.5 μM) in a dose-dependent manner ($p < 0.001$ and $p < 0.001$, respectively) (Fig. 3).

Involvement of TGF-β signaling in the effects of AGE3 on the expression of sclerostin and RANKL in MLO-Y4-A2 cells

Real-time PCR showed that 200 μg/mL AGE3 significantly increased the expression of *Sost* mRNA after 24 h treatment ($p < 0.01$), and that co-incubation with 2.5 μM SD208 significantly decreased the AGE3-increased expression of *Sost* ($p < 0.001$) (Fig. 4A). Moreover, Western blotting showed that co-incubation of SD208 with AGE3 significantly suppressed the AGE3-increased expression of sclerostin protein after treatment for 48 h (Fig. 4B and 4C). In contrast, real-time PCR showed that not only AGE3 but also SD208 significantly decreased the expression of *Rankl* mRNA ($p < 0.01$ and $p < 0.05$, respectively), and that co-incubation of SD208 with AGE3 significantly decreased the expression of *Rankl* mRNA ($p < 0.001$) (Fig. 5A). Moreover, the ratio of RANKL and OPG, a marker for osteoclastic recruitment, was significantly decreased by AGE3 as well as SD208 (Fig. 5B). RANKL ELISA showed that AGE3 significantly decreased the expression of RANKL ($p < 0.05$), and that co-incubation of SD208 with AGE3 also significantly decreased the expression of RANKL ($p < 0.01$) (Fig. 5C). Furthermore, Western blotting showed that AGE3 and SD208 significantly suppressed the expression of RANKL (Fig. 5D and 5E).

Discussion

In this study, we showed that AGE3 increased apoptosis and the expression of sclerostin via TGF-β expression and secretion in MLO-Y4-A2 cells. Moreover, co-incubation with an inhibitor of the TGF-β receptor reduced the effects of AGE3 on apoptosis and sclerostin expression, suggesting that the effects of AGE3 on apoptosis and the expression of sclerostin depend on the enhancement of TGF-β expression by AGE3. However, the inhibition of TGF-β signaling decreased RANKL expression and did not alter the effect of AGE3 on RANKL expression, suggesting that TGF-β signaling itself increases RANKL expression and that TGF-β signaling is not involved in the AGE3-induced suppression of RANKL expression.

TGF-β is a multifunctional polypeptide that regulates a variety of cellular functions, including cell proliferation, differentiation, migration, and apoptosis under both physiological and pathological conditions [25]. TGF-β is sequestered at high levels in bone matrix and is a critical regulator of osteogenesis [26]. Osteoprogenitor differentiation is increased by TGF-β, but matrix mineralization of mature osteoblasts is decreased by TGF-β. In recent years, several studies using *in vivo* animal models have shown that increased TGF-β signaling in bone resulted in induced bone fragility [27-30]. Mice with elevated TGF-β signaling showed decreased bone formation [27, 28], and inhibition of TGF-β signaling rescued the suppressed osteoblast differentiation and bone formation [29, 30]. Our previous study showed that AGE3 increased the expression and secretion of TGF-β in osteoblastic cells [17]. In addition, the present study demonstrates that AGE3 increases TGF-β expression in osteocytic cells, which are the most abundant cells in bone. Taken together, these findings suggest that the detrimental effects of AGEs on bone may be mediated by overexpression of TGF-β.

The present study shows that AGE3 promotes apoptosis by increasing TGF-β expression in MLO-Y4-A2 cells. Skeletal unloading was associated with increased osteocyte apoptosis [31], and mechanical loading repressed the activity of the TGF-β pathway in osteocytes [32]. Although there is no evidence that TGF-β directly

247 induces osteocyte apoptosis, previous studies [31, 32] and our present findings suggest that TGF-β signaling plays
248 important roles in the apoptosis of osteocytes. On the other hand, apoptosis of osteocytes is reported to be closely
249 associated with bone fragility [33, 34]. Previously, clinical studies using high-resolution peripheral quantitative
250 computed tomography showed that cortical porosity might compromise bone mechanical properties and increase
251 fracture risk in T2DM patients who have suffered bone fractures [35]. Thus, AGE-induced apoptosis of osteocytes
252 may contribute to increased cortical porosity in T2DM, although the association between AGE-induced TGF-β
253 signaling and cortical porosity is still unknown.

254 We found that AGE3 increased sclerostin mRNA and protein expression via increased TGF-β signaling.
255 Several studies have shown that TGF-β stimulates the expression of sclerostin in osteocytes. TGF-β and Activin
256 A increased *Sost* expression via *Sost* bone enhancer ECR5 in the UMR106.01 rat osteosarcoma cell line [22].
257 Furthermore, mechanical loading regulated bone formation and sclerostin expression through TGF-β signaling
258 [32]. These findings suggest that sclerostin expression induced by AGE3 induction of the TGF-β signaling
259 pathway might be important for decreased bone formation and increased risk of fractures, which is often seen in
260 diabetic conditions. Indeed, we previously showed that elevated serum levels of sclerostin were associated with
261 high risk of vertebral fractures in patients with T2DM [36]. Although further studies are needed, the inhibition of
262 AGE-induced TGF-β signaling may be a candidate for protecting against T2DM-related bone fragility.

263 RANKL is known to play important roles in osteoclast differentiation and bone resorption. Recent
264 studies have shown that osteocytes express much higher levels of RANKL and have a great capacity to support
265 osteoclastogenesis [37]. Therefore, the mechanism of RANKL expression and its associated regulatory factors in
266 osteocytes are important issues to examine. The present study indicates that TGF-β signaling is not involved in
267 the effects of AGE3 on RANKL expression as well as RANKL to OPG ratio in MLO-Y4-A2 cells. However, it
268 is interesting to note that TGF-β receptor inhibitor alone significantly decreases the expression of RANKL. These

findings suggest that endogenous TGF-β may increase or maintain the expression of RANKL in osteocytes. Previous studies showed that TGF-β decreased RANKL expression in osteoblastic cells, while it increased OPG expression [38]. In this study, OPG expression didn't affected by AGE3. To our knowledge, there are no studies on the direct effects of TGF-β on RANKL expression in osteocytes; therefore, future studies to address this issue are necessary.

We examined the effect of AGE3 on expression of RAGE, which is a cell surface receptor that belongs to the immunoglobulin superfamily and play an important role in the action of AGEs [39]. Also, we have previously reported that knockdown of Rage by siRNA significantly blunted the effects of AGEs on the expression of sclerostin and RANKL in MLO-Y4-A2 cells [18]. Furthermore, the present study revealed that AGE3 slightly but significantly increased the expression of RAGE. These findings suggest that there is a vicious cycle that AGE3-RAGE signaling induces RAGE upregulation which in turn enhances further influence of AGE3 and subsequent RAGE overexpression. However, several types of AGEs binding proteins have been identified in various cells [40]. For example, Toll-like receptors are reported to be involved in AGEs action [41, 42]. Although we focused on the AGE3-TGF-β signaling pathway in the present study, previous studies showed that other proinflammatory cytokines such as tumor necrosis factor-α and interleukins in rat mesenchymal stem cells and human osteoblast cells [43, 44]. Therefore, further examination should be necessary to clarify the underlying mechanism of AGEs-induced dysfunction of osteocytes in future.

Conclusions

The present study indicates that AGE3 increases apoptosis and sclerostin expression by increasing TGF-β expression and secretion in MLO-Y4-A2 cells, and that TGF-β signaling is not involved in the AGE3-induced suppression of RANKL expression in these cells. AGE3-induced TGF-β signaling may be associated

with the AGE-induced bone fragility and elevated fracture risk of T2DM patients via an increase in sclerostin expression and osteocyte apoptosis. To extend the present findings, further in vivo experiments are needed in the future.

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Figure legends

Fig. 1. Effects of AGE3 on *Sost* and *Rankl* mRNA expression and apoptosis of MLO-Y4-A2 cells

Bovine serum albumin (BSA, control: 200 μg/mL) or AGE3 (200 μg/mL) was added after the cells reached confluence. (A) Effect of AGE3 on the apoptotic cell death of MLO-Y4-A2 cells. The cells were treated with 200 μg/mL of either control BSA or AGE3 for 5 days. Apoptotic cell death was analyzed by ELISA for DNA fragments. ***$p < 0.001$ compared to control BSA. (B) (C) Total RNA was collected on day 1, and the mRNA expression of *Sost* and *Rankl* was examined by quantitative real-time PCR. Results are expressed as the mean fold increase over control values ± SEM ($n = 6$). **$p < 0.01$ compared to control BSA.

Fig. 2. Effects of AGE3 on *Tgf-β* and *Rage* mRNA and protein expression in MLO-Y4-A2 cells

Bovine serum albumin (BSA, control: 200 μg/mL) or AGE3 (200 μg/mL) was added after the cells reached confluence. (A) Effect of AGE3 on *Tgf-β* mRNA expression in MLO-Y4-A2 cells. Total RNA was collected at 6, 12 and 24 h, and the expression of mRNA was examined by real-time PCR. Results are expressed as the mean fold increase over control values ± SEM ($n = 10$). *$p < 0.05$ and **$p < 0.01$ compared to control at the same time point. (B) Effects of AGE3 on TGF-β protein expression in MLO-Y4-A2 cells. Whole cell lysates were collected on days 1, 3, and 5. Quantification of TGF-β protein was performed by an ELISA kit. Results are expressed as the mean ± SEM ($n = 6$). ***$p < 0.001$ compared to control on the same day. (C) Effect of AGE3 on *Rage* mRNA expression in MLO-Y4-A2 cells for 1 day.

Fig. 3. Effects of a TGF-β receptor kinase inhibitor on AGE3-induced apoptotic cell death of MLO-Y4-A2 cells

After the cells reached confluence, the cells were treated with 200 μg/mL of either control BSA or AGE3 in the

447 presence or absence of SD208 for 5 days. Apoptotic cell death was analyzed in an ELISA for DNA fragments.
448 *p < 0.05 and ***p < 0.001
449
450 **Fig. 4. Effects of a TGF-β receptor kinase inhibitor on AGE3-induced *Sost* mRNA and sclerostin protein**
451 **expression in MLO-Y4-A2 cells**
452 Control BSA (200 μg/mL) or AGE3 (200 μg/mL) with or without SD208 (2.5 mM), a TGF-β type I receptor
453 kinase inhibitor, were added after the cells reached confluence. (A) Total RNA was collected on day 1, and *Sost*
454 mRNA expression was examined by quantitative real-time PCR. Results are expressed as the mean fold increase
455 over control values ± SEM (n = 6). **p < 0.01, and ***p < 0.001. (B and C) Western blot analysis for sclerostin
456 and β-actin was performed on total protein extracts on day 2. The representative picture is shown in B and
457 quantification and statistical analysis are shown in C.
458
459 **Fig. 5. Effects of a TGF-β receptor kinase inhibitor on *Rankl* mRNA and RANKL protein expression in**
460 **the presence and absence of AGE3 in MLO-Y4-A2 cells**
461 Control BSA (200 μg/mL) or AGE3 (200 μg/mL) with or without SD208 (2.5 mM), a TGF-β type I receptor
462 kinase inhibitor, were added to confluent cells. (A) Total RNA was collected on day 1, and *Rankl* mRNA
463 expression was examined by real-time PCR. Results are expressed as the mean fold increase over control values
464 ± SEM (n = 6). *p < 0.05, **p < 0.01, and ***p < 0.001 compared to control. (B) The ratio of *Rankl* to *Opg*
465 mRNA expression was examined by real-time PCR. (C) Whole cell lysates were collected on day 2.
466 Quantification of RANKL was performed by an ELISA kit. Results are expressed as the mean ± SEM (n = 6).
467 *p < 0.05, and **p < 0.01 compared to control. (D and E) Western blot analysis for RANKL and β-actin was
468 performed on total protein extracts on day 2. The representative picture is shown in D and quantification and

469 statistical analysis are shown in E.