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Glycolaldehyde-modified advanced glycation end-products inhibit differentiation of human monocytes into osteoclasts via upregulation of IL-10

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ABSTRACT

Diabetes patients are at high risk of bone fracture due to accumulation of advanced glycation end products (AGEs) and low bone turnover. Although AGEs inhibit osteoblast functions, little is known about their roles in regulation of human osteoclast differentiation. The aim of this study was to determine the roles of AGEs in regulation of human osteoclast differentiation. Human $CD14^+$ monocytes collected from healthy individuals were stimulated in vitro with conventional cytokines to induce osteoclast differentiation. Simultaneously, glucose-modified AGEs-BSA (Glu-AGEs-BSA) and glycolaldehyde-modified AGEs-BSA (Glyco-AGEs-BSA) were added to analyze their role in regulation of osteoclast differentiation. Human $CD14^+$ cells expressed endogenous receptor for AGE (RAGE). Stimulation with Glyco-AGEs-BSA, but not Glu-AGEs-BSA, reduced the number of tartrate-resistant acid phosphatase-positive cells in a dose-dependent manner and suppressed mRNA expression of *nuclear factor of activated T-cells 1* and *cathepsin K*. Glyco-AGEs-BSA up-regulated pro-inflammatory cytokines and anti-inflammatory cytokine IL-10. The addition of IL-10-neutralizing antibodies abrogated the suppressive effect of Glyco-AGEs-BSA on osteoclast differentiation. Stimulation of Glyco-AGE-BSA resulted in nuclear factor (NF)-κB phosphorylation, and addition of an inhibitor of κB kinase suppressed IL-10 production. We conclude that Glyco-AGEs-BSA inhibited human osteoclast differentiation through induction of IL-10 expression via NF-κB. It can be assumed that AGE bioaccumulation in diabetic patients increases the risk of bone fracture, through inhibition of osteoclast differentiation, reduction of bone turnover, and disruption of bone remodeling.

1. Introduction

Patients with diabetes are at higher risk of vasculopathies, including micro- and macro-vascular diseases, which are associated with the progression of arteriosclerosis [1]. In addition, diabetics are at higher risk of reduced bone turnover and associated with a complication of osteoporosis and bone fracture [2–5].

One of the possible reasons for the increased risk of bone fracture is accumulation of advanced glycation end-products (AGEs), which are produced non-enzymatically by the reaction of reducing sugars (e.g., glucose) with proteins, which is related to the persistent hyperglycemic state [6]. After the binding of AGEs to their receptor (RAGE), AGEs can potentially produce cellular dysfunction by intracellular signals, including intracellular reactive oxygen species, mitogen-activated protein kinase, and the transcription factor nuclear factor (NF)-κB [7,8]. Accumulation of AGEs in bone tissue increases non-physiological AGE cross-linking in the bone, which in turn reduces bone strength, without affecting bone mineral density [9]. Some studies have indicated that pentosidine, a type of AGEs, is associated with the risk of bone fracture in patients with type 2 diabetes [10]. Regarding their role in bone metabolism, AGEs are known to disrupt osteoblast function and inhibit osteoblast differentiation by inducing apoptosis [11]. Glycolaldehyde-modified AGEs (Glyco-AGEs) reportedly increase expression of transforming growth factor (TGF)-β in human mesenchymal stem cells and inhibit calcification [12]. Although the mechanism by which AGEs inhibit bone formation has been partially elucidated, no consensus has

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been reached on the significance of AGEs in osteoclast differentiation or bone resorption [13–16]. To our knowledge, there is only a single study that has described the effects of AGEs on human osteoclasts. The elucidation of role of AGEs in human bone metabolism should contribute not only to basic research but also to finding new therapeutic targets for correcting abnormal bone metabolism in patients with diabetes. Therefore, we investigated the action mechanism of AGEs in human osteoclast differentiation in vitro.

2. Materials and methods

2.1. Cell cultures and stimulatory experiments

Human $CD14^+$ cells were isolated from peripheral blood mononuclear cells (PBMCs) obtained from healthy donors who were randomly selected by exhaustive immunomagnetic positive selection. In all experiments, we used AutoMACS separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany) and $CD14^+$ MicroBeads (Miltenyi Biotec). $CD14^+$ cells were seeded into a 96-well plate (Corning, Tokyo, Japan) at a density of 1×10^5 cells/well, and cultured in α-MEM medium (Gibco Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (FCS, Nichirei Biosciences, Tokyo) and 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA) at 37 °C in a 5% CO_2 atmosphere. Osteoclast differentiation was stimulated as described previously [17]. Briefly, human $CD14^+$ cells were differentiated into osteoclast precursor cells in the presence of 25 ng/ml human macrophage colony-stimulating factor (25 ng/ml, M-CSF; PeproTech, Rocky Hill, NJ) from culture days 0 to 3. Osteoclast precursor cells differentiated into mature osteoclasts in the presence of M-CSF and human soluble receptor activator of nuclear factor-κB ligand (100 ng/ml, sRANKL; PeproTech) from culture days 3 to 12. Culture media were replaced every three days. To determine the effects of AGEs, $CD14^+$ cells were treated with glucose-modified AGEs-BSA (100 μg/ml, Glu-AGEs-BSA; BioVision, Mountain View, CA), glycolaldehyde-modified AGEs-BSA (100 μg/ml, Glyco-AGEs-BSA; BioVision), or Control-BSA (100 μg/ml, BioVision) from culture days 0 to 12. Recombinant human IL-10, neutralizing antibody against human IL-10, and isotype control were purchased from R&D Systems (Minneapolis, MN). IKK inhibitor (Bay11-7082), JNK inhibitor (SP100625), and ERK inhibitor (SCH772984) were purchased from Abcam (Cambridge, MA), Calbiochem (San Diego, CA), and Selleckchem (Houston, TX), respectively.

2.2. Tartrate-resistant acid phosphatase (TRAP) staining

Osteoclast differentiation was confirmed using an Acid Phosphatase Leukocyte Kit (Sigma, San Diego, CA) according to the instructions provided by the manufacturer. TRAP-positive, multi-nucleated cells that contained three or more nuclei were counted as osteoclasts.

2.2.1. Quantitative reverse transcription (RT-q) PCR

RT-qPCR was conducted as described previously [18]. Briefly, total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed using SuperScript®VILO™ master mix (Life Technologies). qPCRs were run on a Step One Plus system (Applied Biosystems) using TaqMan®Fast Universal PCR master mix (Applied Biosystems). The following TaqMan® Gene Expression Assay (Applied Biosystems) primer/probe pairs were used: *nuclear factor of activated T-cells 1* (*NFATc1*, Hs00542678_m1), *cathepsin K* (*CTSK*, Hs01080388_m1), *interleukin-10* (*IL-10*, Hs00961622_m1), and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*, Hs99999905_m1).

2.3. Measurement of cytokine levels in culture supernatants

$CD14^+$ cell culture supernatants were collected. The levels of osteoclastogenic cytokines, including tumor necrosis factor-α (TNF-α), IL-1β, and IL-6, and anti-osteoclastogenic cytokines, including IL-4, IL-10, IL-12/p70, interferon-α (IFN-α), and IFN-γ, in the supernatants were measured using a BD Cytometric Bead Array Human Flex Set (BD Pharmingen, San Diego, CA) according to the instructions supplied by the manufacturer [19]. Fluorescence data were analyzed using the BD Cytometric Bead Array Analysis software.

2.4. Immunoblotting

Immunoblotting was carried out as described previously [20], with slight modification. Briefly, whole cell extracts were lysed in TNE buffer [50 mM Tris-HCl (pH 7.4), 0.5% (*v*/v) nonidet P-40,150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, and 10 mg/ml aprotinin]. Equal amounts of protein (7 μg) were separated by SDS-PAGE, subjected to blocking, and then transferred onto nitrocellulose (Protran BA83; GE Healthcare, Chalfont St. Giles, UK). The membranes were immunoblotted with anti-phospho-NF-κB p65 [Ser 536, #3033; Cell Signaling Technology (CST), Danvers, MA], anti-NF-κB p65 (#8242; CST), anti-RAGE (sc-365,154; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-actin (A1978; Sigma-Aldrich, St. Louis, MO) antibodies. Bound antibodies were visualized with horseradish peroxidase-labeled anti-secondary antibodies against mouse or rabbit IgG (GE Healthcare) using chemiluminescence reagents (ECL™ Prime Western Blotting Detection Reagent; GE Healthcare).

2.5. Statistical analysis

For in-vitro experiments, data were expressed as mean ± standard error of the mean (SEM) of at least three independent experiments using different donors. Differences between groups were examined by the Student's *t*-test. In all analyses, a P-value < 0.05 was considered significant. All statistical analyses were conducted using Statistical Package for Social Sciences software, version 25.0 (SPSS, Chicago, IL).

2.6. Ethics approval

The institutional review board of the University of Occupational and Environmental Health Japan approved the study and volunteers signed a consent form in accordance with the Declaration of Helsinki.

3. Results

3.1. Human $CD14^+$ cells express endogenous RAGE

To investigate whether human $CD14^+$ cells (monocytes) express endogenous RAGE, monocytes were isolated from PBMCs, treated with or without 25 ng/ml M-CSF on day 0, and then stimulated with Glu-AGEs-BSA (100 μg/ml), Glyco-AGEs-BSA (100 μg/ml) or control-BSA (100 μg/ml) for 24 h. Whole-cell lysates of monocytes or A549 cells were prepared and analyzed by immunoblotting with anti-RAGE and anti-β-actin antibodies. These studies showed clear expression of RAGE on monocytes, but the expression level was neither altered by Glu-AGEs-BSA nor Glyco-AGEs-BSA. Upregulation of RAGE was also observed on A549 cells, a human pulmonary epithelial cell line [21] (Fig. 1).

3.2. Glyco-AGEs-BSA inhibits differentiation of human monocytes into osteoclasts

Human $CD14^+$ cells were stimulated with M-CSF and sRANKL for differentiation into TRAP-positive multi-nucleated osteoclasts. Whereas neither Glu-AGEs-BSA nor Control-BSA affected osteoclast differentiation, Glyco-AGEs-BSA inhibited osteoclast differentiation in a dose-dependent manner (Fig. 2A). Furthermore, mRNA expression of *NFATc1*, which encodes a master regulator of osteoclastogenesis, was enhanced upon stimulation with M-CSF and sRANKL on day 6. The addition of

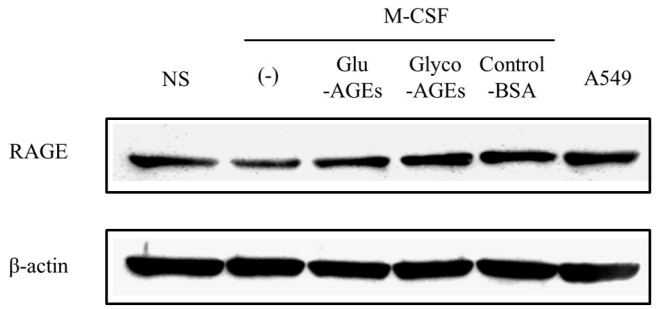

Fig. 1. Human CD14$^+$ cells express endogenous RAGE.
Human CD14$^+$ cells (monocytes) were isolated from PBMCs, treated with or without 25 ng/ml M-CSF on day 0, and then stimulated with Glu-AGEs-BSA (100 μg/ml), Glyco-AGEs-BSA (100 μg/ml) or control-BSA (100 μg/ml) for 24 h. Whole-cell lysates of monocytes or A549 cells were prepared and analyzed by immunoblotting with anti-RAGE and anti-β-actin antibodies. Representative data of three independent experiments were shown. RAGE, receptor for advanced glycation end products; PBMCs, peripheral blood mononuclear cells; M-CSF, macrophage-colony stimulating factor; Glu-AGEs, glucose-modified-AGEs; Glyco-AGEs, glycolaldehyde-modified AGEs.

Glyco-AGEs-BSA, but not Glu-AGEs-BSA, significantly inhibited *NFATc1* mRNA expression (Fig. 2B). Likewise, *CTSK* mRNA expression was upregulated by stimulation with M-CSF and sRANKL on day 6, but significantly inhibited by stimulation with Glyco-AGEs-BSA (Fig. 2B).

3.3. Glyco-AGEs-BSA induces IL-10 production during osteoclast differentiation

To investigate the mechanism by which AGEs inhibit osteoclast differentiation, the levels of those cytokines associated with osteoclast differentiation were quantified in cell culture supernatants. In the early stage of osteoclast differentiation (day 1), Glyco-AGEs-BSA increased the levels of all osteoclastogenic cytokines evaluated (TNF-α, IL-1β, and IL-6) (Fig. 3A). Furthermore, Glyco-AGEs-BSA significantly increased the levels of IL-10 (Fig. 3A), but not other anti-osteoclastogenic cytokines, such as IL-4, IL-12/p70, IFN-α, and IFN-γ (data not shown). In the middle stage of osteoclast differentiation (day 6), Glyco-AGEs-BSA had no effect on TNF-α and IL-1β levels, but it significantly increased IL-6 and IL-10 levels in the supernatant (Fig. 3B). In the late stage of osteoclast differentiation (day 9), Glyco-AGEs-BSA had little effect on IL-6 level, but still significantly increased IL-10 level (Fig. 3C).

To investigate the potential association between IL-10 and TNF-α, IL-1β, and IL-6 levels, CD14$^+$ monocytes were stimulated with recombinant IL-10, and the levels of TNF-α, IL-1β, and IL-6 were measured separately. IL-10 suppressed the levels of TNF-α, IL-1β, and IL-6 in a dose-dependent manner (Fig. 3D). These results suggest that IL-10, persistently induced by Glyco-AGEs-BSA, can inhibit the production of TNF-α, IL-1β, and IL-6.

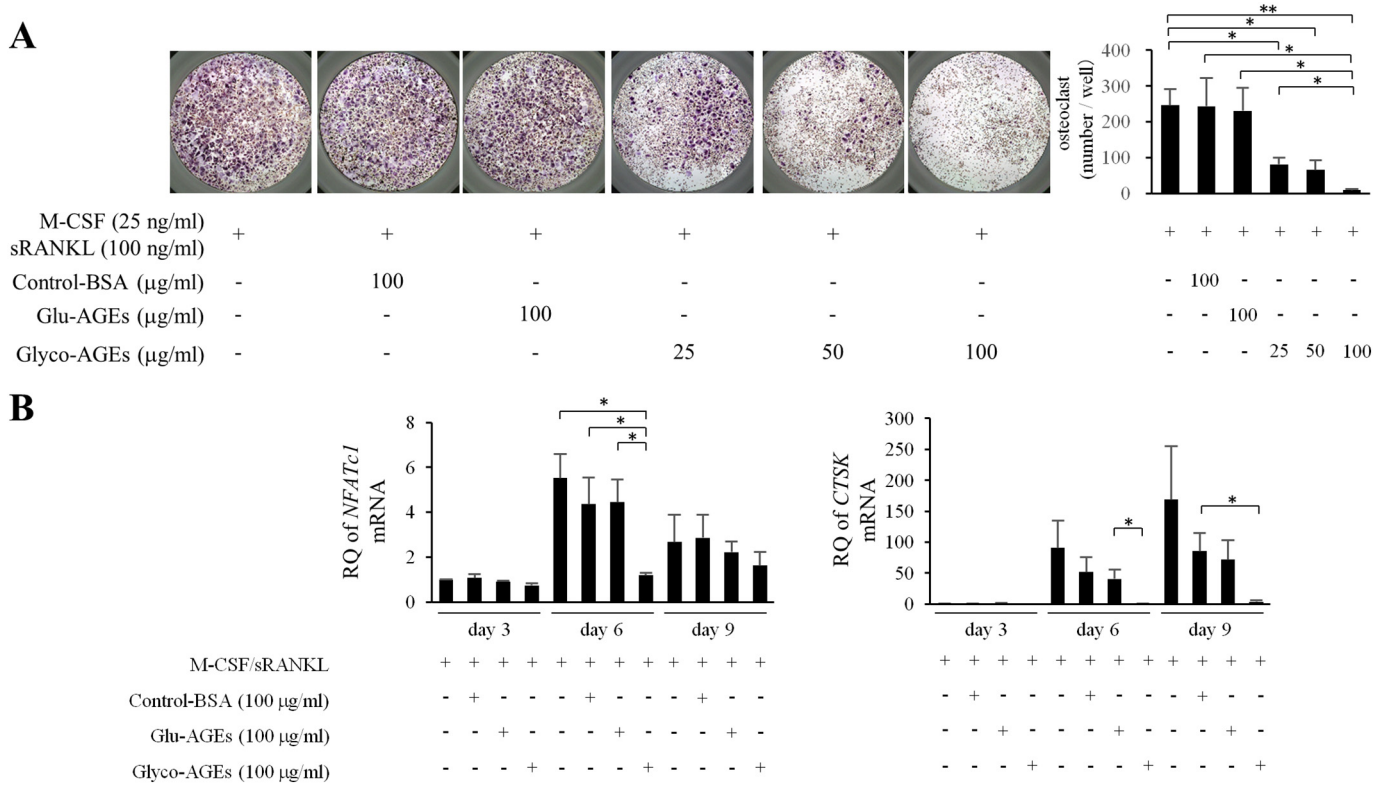

Fig. 2. Glyco-AGEs-BSA inhibits the differentiation of human monocytes into osteoclasts via suppression of mRNA expression of *NFATc1* and *CTSK*.
Human CD14$^+$ cells (monocytes) were isolated from PBMCs and treated with M-CSF 25 ng/ml on days 0, 3, 6 and 9, and sRANKL 100 ng/ml on days 3, 6 and 9. (A) Glu-AGEs-BSA (100 μg/ml), Glyco-AGEs-BSA (25 to 100 μg/ml) or control-BSA (100 μg/ml) was added to monocytes on day 0. TRAP staining was performed on day 12. Representative data of three independent experiments were shown. TRAP-positive and multi-nucleated cells that contained three or more nuclei were counted as osteoclasts and counted on day 12. Data are mean ± SEM (n = 3). $^*P < 0.05$, $^{**}P < 0.01$ by the Student's t-test. (B) Cells were lysed and total RNA was isolated on days 3, 6, and 9. The mRNA expression of *NFATc1* and *CTSK* was quantified by RT-qPCR and normalized to *GAPDH*. Data are mean ± SEM (n = 5). $^*P < 0.05$ by the Student's t-test. PBMCs, peripheral blood mononuclear cells; M-CSF, macrophage-colony stimulating factor; sRANKL, soluble receptor activator of nuclear factor-κB ligand; Glu-AGEs, glucose-modified-AGEs; Glyco-AGEs, glycolaldehyde-modified AGEs; TRAP, tartrate-resistant acid phosphatase; NFATc1, nuclear factor of activated T-cells 1; CTSK, cathepsin K; GAPDH; glyceraldehyde-3-phosphate dehydrogenase.

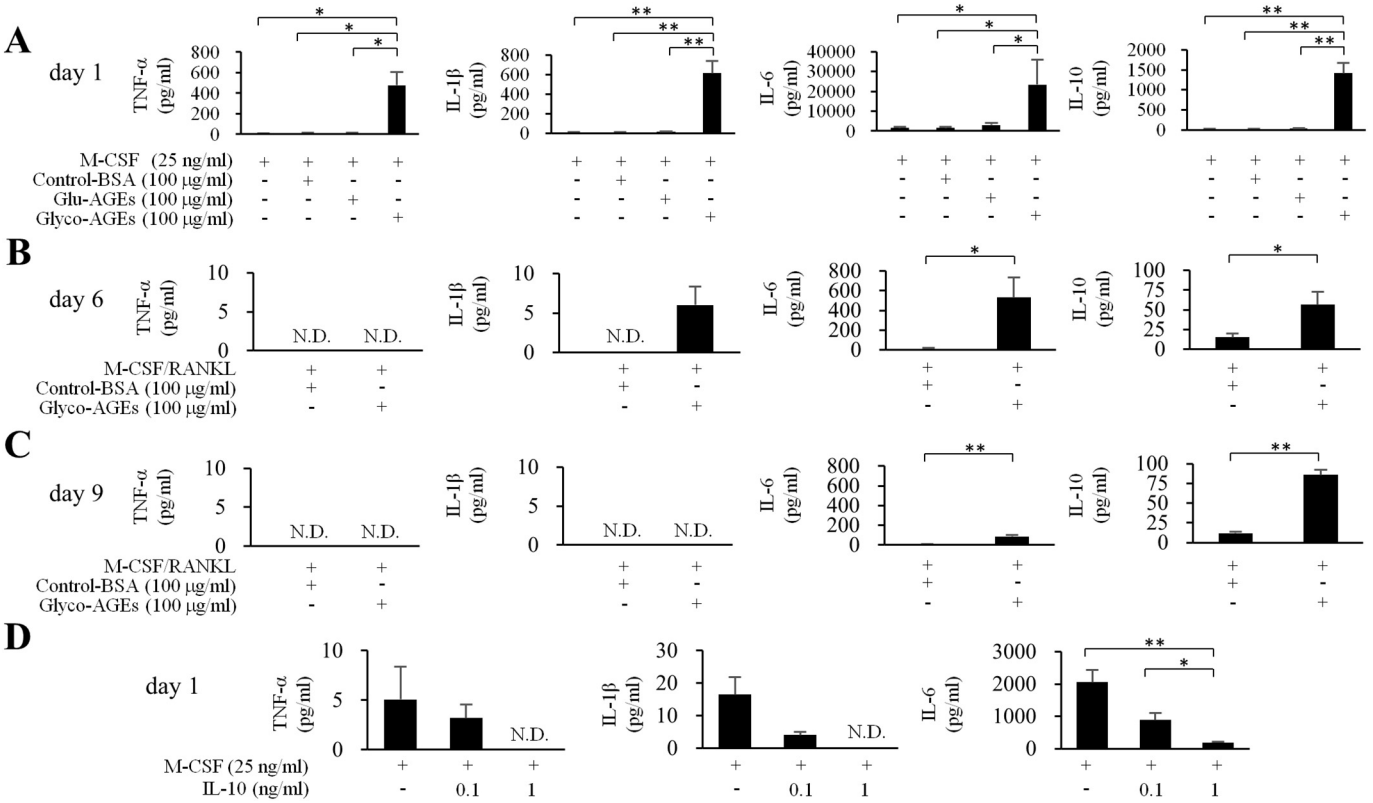

Fig. 3. Glyco-AGEs-BSA induces IL-10 production and then suppresses TNF-α, IL-1β, and IL-6 production during osteoclast differentiation.
Culture supernatants were collected from CD14$^+$ cells. Osteoclastogenic cytokines, such as TNF-α, IL-1β and IL-6, and anti-osteoclastogenic cytokines, such as IL-4, IL-10, IL-12/p70, IFN-α, and IFN-γ were measured in supernatants collected on day 1 using a BD Cytometric Bead Array Human Flex Set. (A–C) TNF-α, IL-1β, IL-6, and IL-10 levels were measured on day 1 (A), day 6 (B), and day 9 (C) in the absence or presence of Glu-AGEs-BSA (100 μg/ml), Glyco-AGEs-BSA (100 μg/ml), or control-BSA (100 μg/ml), with M-CSF (25 ng/ml) and sRANKL (100 ng/ml). (D) TNF-α, IL-1β, and IL-6 levels measured on day 1 in the presence or absence of recombinant IL-10 (0.1 or 1 ng/ml), with M-CSF (25 ng/ml). Data are mean ± SEM ($n = 4$). $^*P < 0.05$, $^{**}P < 0.01$ by the Student's *t*-test. Glu-AGEs, glucose-modified-AGEs; Glyco-AGEs, glycolaldehyde-modified AGEs; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; CBA, Cytometric Bead Array; M-CSF, macrophage-colony stimulating factor.

3.4. Neutralizing antibody against IL-10 antagonizes the inhibitory effect of Glyco-AGEs-BSA on osteoclastogenesis

Next, we examined the effects of IL-10 on osteoclast differentiation. The addition of IL-10 suppressed human osteoclast differentiation or reduced the number of osteoclasts in a dose-dependent manner (Fig. 4A). IL-10 significantly inhibited *NFATc1* mRNA expression on day 6 (Fig. 4B). Whereas osteoclast differentiation was inhibited by Glyco-AGEs-BSA, such inhibition was partially abrogated in the presence of IL-10-neutralizing antibody, but not isotype control antibody (Fig. 5).

3.5. IKK-inhibitor bay11-7082 suppresses Glyco-AGEs-BSA-induced IL-10 mRNA expression

To further investigate the mechanism by which Glyco-AGEs-BSA induces IL-10, human CD14$^+$ cells were stimulated for 30 min with M-CSF in combination with Glu-AGEs-BSA, Glyco-AGEs-BSA, or Control-BSA. The expression level of NF-κB and its phosphorylation status downstream of AGE signaling were analyzed by immunoblotting. Glyco-AGEs-BSA did not alter the expression level of total NF-κB p65, but significantly upregulated its phosphorylation, compared with Control-BSA (Fig. 6A). These results suggest that Glyco-AGEs-BSA seems to modulate IL-10 level by transmitting signals via NF-κB p65 phosphorylation in human CD14$^+$ cells. When the same cells were exposed for 5 h to both Glyco-AGEs-BSA and bay11-7082, a IKK inhibitor, *IL-10* mRNA expression was significantly reduced in a IKK inhibitor dose-dependent manner (Fig. 6B). In contrast, the addition of JNK 1/2/3 inhibitor or ERK 1/2 inhibitor together with Glyco-AGEs-BSA had little effect on mRNA expression of *IL-10* compared with Glyco-AGEs-BSA alone. These findings suggest that Glyco-AGEs-BSA seems to induce IL-10 expression via phosphorylation of NF-κB.

4. Discussion

The complications of diabetes include micro- and macro-vasculopathies, as well as low bone turnover with associated osteoporosis and bone fragility [2,4,5]. The present study demonstrated that Glyco-AGEs, a type of AGEs produced under chronic hyperglycemia, inhibit human osteoclast differentiation through IL-10 signaling pathway in vitro.

RAGE, a receptor for AGEs, is expressed in a wide range of tissues containing pulmonary epithelial cells, vascular endothelial cells, vascular smooth muscle cells, vascular pericytes, renal mesangial cells, macrophages, T-lymphocytes, monocytes, and osteoblasts [21–27]. A recent study indicated that Glycer-AGEs and Glyco-AGEs of various AGE compounds induced angiogenesis via RAGE [28]. In mice, AGEs up-regulate both inflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6) and the anti-inflammatory cytokine IL-10 in monocytes [29,30]. In human, stimulation of monocytes with AGEs enhances the production of TNF-α, IL-1β, and IL-6 [24,31]. The present study demonstrated for the first time that Glyco-AGEs also enhance the production of IL-10 in human monocytes. In other words, Glyco-AGEs amplify signals from TNF-α, IL-1β, and IL-6, which promote osteoclast differentiation, as well as signals from IL-10, which inhibits osteoclast differentiation from human monocytes. Of note, IL-10 production was persistently enhanced by Glyco-AGEs, whereas that of TNF-α, IL-1β, and IL-6 was enhanced

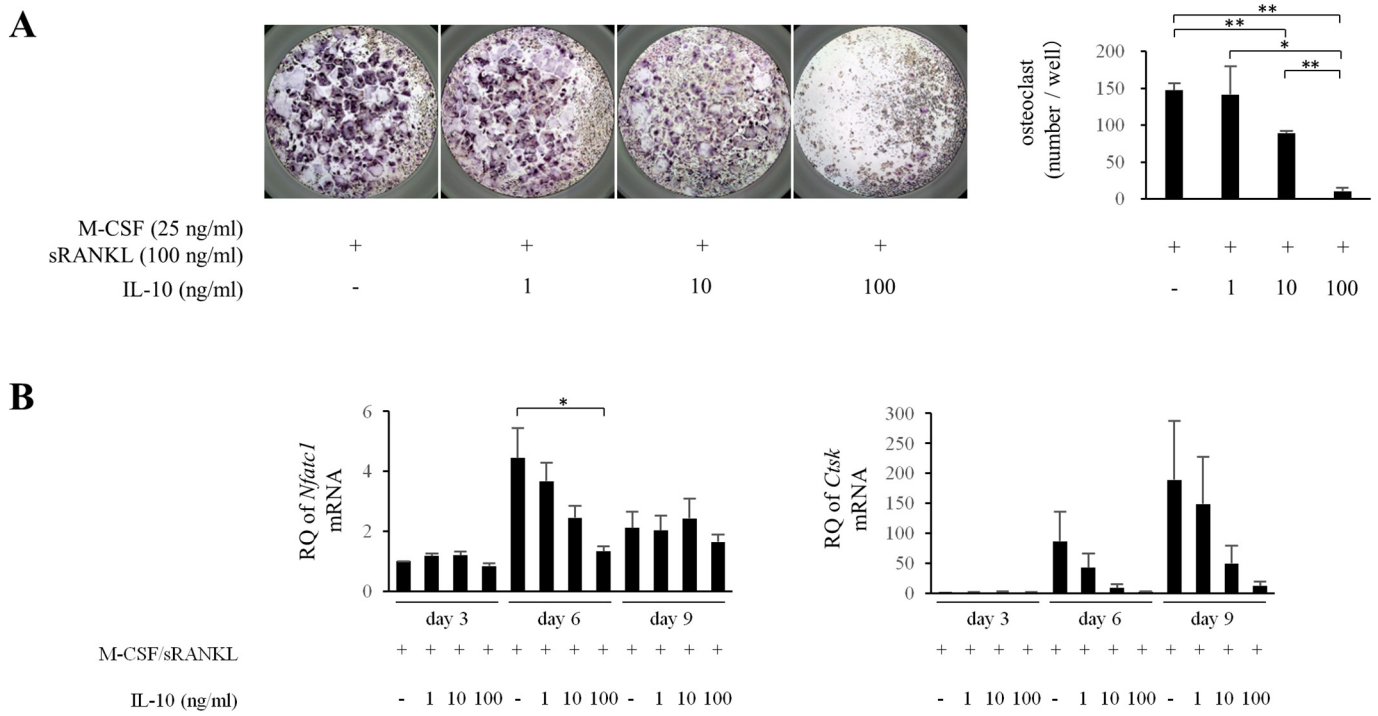

Fig. 4. Recombinant human IL-10 inhibits human osteoclast differentiation and mRNA expression of *NFATc1* and *CTSK*.
Human CD14$^+$ cells isolated from PBMCs were treated with M-CSF 25 ng/ml on days 0, 3, 6, and 9, and with sRANKL 100 ng/ml on days 3, 6, and 9. (A) Monocytes were treated on day 0 with recombinant IL-10 (1 to 100 ng/ml). TRAP staining was performed and osteoclasts were counted on day 12 as described in Fig. 2. Representative data of three independent experiments are shown. Data are mean ± SEM ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$ by the Student's *t*-test. (B) Cells were lysed and total RNA was isolated on days 3, 6, and 9, as described in Materials and Methods. The mRNA expression of *NFATc1* and *CTSK* was measured by RT-qPCR and normalized to *GAPDH*. Data are mean ± SEM (n = 4). $^*P < 0.05$ by the Student's *t*-test. IL, interleukin; PBMCs, peripheral blood mononuclear cells; M-CSF, macrophage-colony stimulating factor; sRANKL, soluble receptor activator of nuclear factor-κB ligand; TRAP, tartrate-resistant acid phosphatase; NFATc1, nuclear factor of activated T-cells 1; CTSK, cathepsin K; GAPDH; glyceraldehyde-3-phosphate dehydrogenase.

over a limited period of time and diminished over time. As one of the underlying mechanisms, the present study showed that IL-10 was strongly induced by Glyco-AGEs, and inhibited autocrine production of TNF-α, IL-1β, and IL-6. With regard to the mechanism by which Glyco-AGEs induced the expression of IL-10, pharmacological inhibition experiments identified NF-κB as one of the factors involved in IL-10 expression by Glyco-AGEs. We conclude that persistent production of IL-10 via NF-κB signals from Glyco-AGEs negatively regulates osteoclast differentiation.

Glyco-AGEs-BSA, but not Glu-AGEs-BSA, inhibited osteoclast differentiation. Previous studies reported that Glyco-AGEs-BSA, but not Glu-AGEs-BSA, increased the expression of RAGE [27]. However, our study showed no difference between the two types of AGEs on RAGE expression level. However, our results showed that stimulation of CD14$^+$ cells with high concentration of Glu-AGEs-BSA (1000 μg/ml) enhanced the production of cytokines, including TNF-α, IL-1β, IL-6, and IL-10 (data not shown). These results suggest that Glu-AGEs-BSA seem to have a lower affinity for RAGE compared with Glyco-AGEs-BSA. Considered together, we conclude that at 100 μg/ml, Glu-AGEs-BSA cannot induce NF-κB activation.

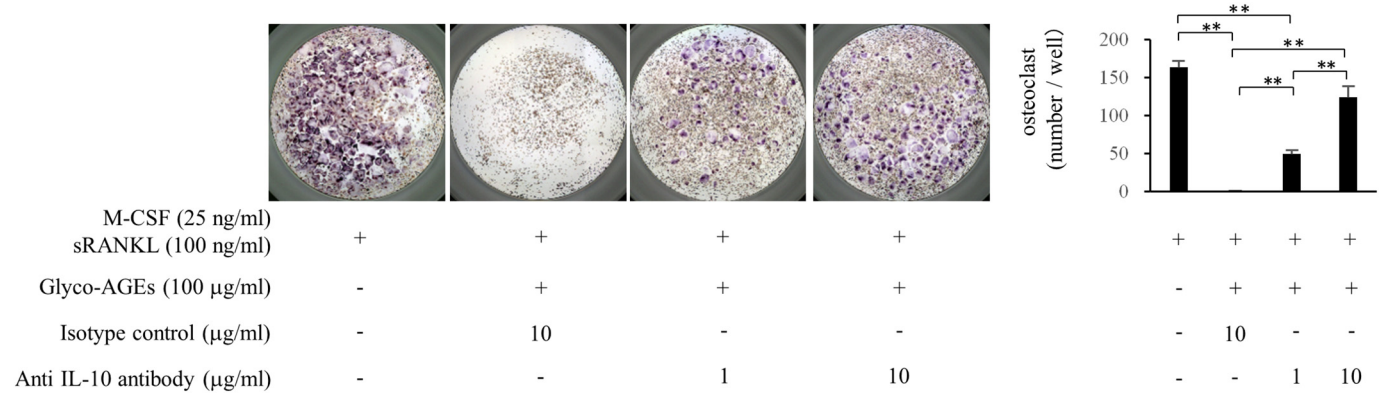

Fig. 5. Neutralizing antibody against IL-10 antagonizes the inhibitory effect of Glyco-AGEs-BSA on osteoclastogenesis.
Monocytes were treated with neutralizing antibody against IL-10 (1 or 10 ng/ml) or isotype control (10 ng/ml) in the presence of Glyco-AGEs-BSA (100 μg/ml). TRAP staining was performed and osteoclasts were counted on day 12 as described in Fig. 2. Representative data of three independent experiments are shown. Data are mean ± SEM (n = 3). $^*P < 0.05$, $^{**}P < 0.01$ by the Student's *t*-test. IL, interleukin; Glyco-AGEs, glycolaldehyde-modified AGEs; TRAP, tartrate-resistant acid phosphatase.

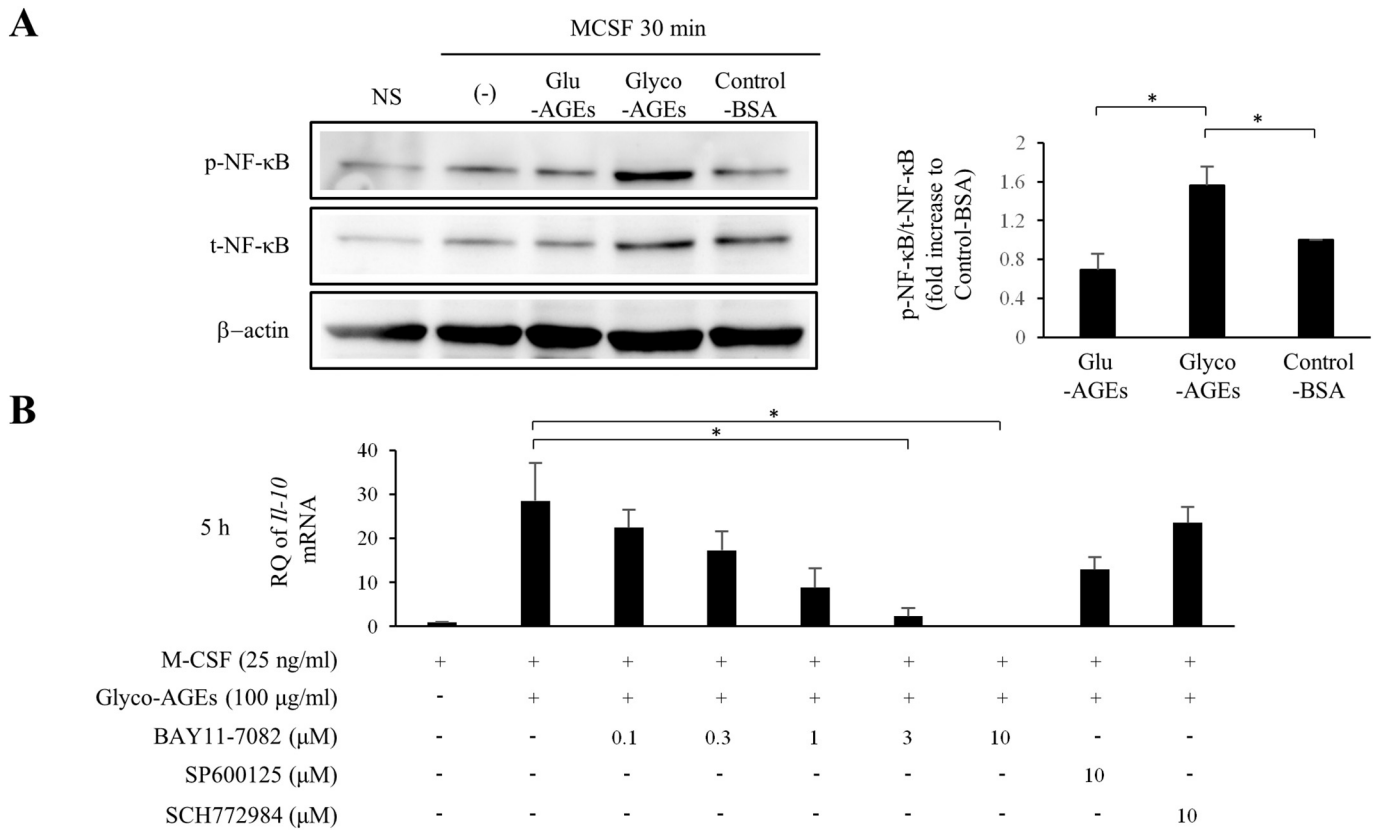

Fig. 6. Glyco-AGEs-BSA up-regulates *IL-10* mRNA via phosphorylated NF-κB p65 in human monocytes.
(A) Human monocytes were pre-incubated for 60 min at 37 °C under 5% CO_2 atmosphere and then treated with M-CSF (25 ng/ml) for 30 min in the presence or absence of Glu-AGEs-BSA (100 μg/ml), Glyco-AGEs-BSA (100 μg/ml), or control-BSA (100 μg/ml). Whole-cell lysates were prepared and analyzed by immunoblotting with anti-phospho-NF-κB p65, anti-NF-κB p65, anti-β-actin antibodies. The right panel represents phospho/total NF-κB ratio of intensities (Control-BSA = 1) Representative data of three independent experiments are shown. Data are mean ± SEM (n = 3). $^{*}P < 0.05$ by the Student's *t*-test. (B) Human monocytes were treated with Glyco-AGEs-BSA (100 μg/ml) in the presence or absence of Bay11-7082 (IKK inhibitor, 0.1–10 μM), SP600125 (JNK inhibitor, 10 μM), or SCH772984 (ERK inhibitor, 10 μM) for 5 h. *IL-10* mRNA expression was quantified by RT-qPCR and normalized to *GAPDH*. Data are mean ± SEM (n = 3). $^{**}P < 0.05$ by the Student's *t*-test. M-CSF, macrophage-colony stimulating factor; Glu-AGEs, glucose-modified AGEs; Glyco-AGEs, glycolaldehyde-modified AGEs; NF-κB, nuclear factor-kappa B; IKK, inhibitor of κB kinase; JNK, c-Jun N-terminal kinase; EKK, extracellular signal-regulated kinase; GAPDH; glyceraldehyde-3-phosphate dehydrogenase, IL, interleukin.

In the human body, serum AGE levels correlate with vascular inflammation and can be used as a biomarker for the severity of arteriosclerosis, whereas their effects on bone metabolism remain unknown [32]. Specifically, AGEs inhibited osteoclast differentiation in vitro, indicating they are, at least in part, responsible for low bone turnover. Thus, AGEs seem to have important effects on bone metabolism, in addition to their well-known effects on arteriosclerotic lesions. This suggests that AGEs can play a role in the bone–vascular axis, in which some authors hypothesized the existence of a close link between osteoporosis and cardiovascular disease [33,34].

There is no established treatment for abnormally low bone turnover in patients with diabetes. Therapeutics targeting IL-10, which inhibits osteoclastogenesis, can simultaneously increase the production of pro-inflammatory cytokines, and thus potentially worsen diabetic vasculopathies. For this reason, inhibition of AGE-RAGE signaling, which is upstream of IL-10, seems to be more appropriate for treatment of abnormal bone metabolism in diabetes. Lifestyle interventions, such as a low-AGE diet and physical exercise, are easy to implement and have beneficial effects on plasma AGEs, leading to reduction of inflammation and oxidative stress. One study showed that replacement of short-acting insulin with glulisine significantly decreased both glycosylated hemoglobin (HbA1c) and serum levels of AGEs in patients with diabetes [35]. Although AGE production inhibitors, RAGE inhibitors, and other drugs have been developed, there is no clear evidence for their efficacy or safety [36,37]. The development of more specific inhibitors is needed in the future.

In conclusion, Glyco-AGEs-BSA inhibited human osteoclast differentiation through induction of IL-10 expression via NF-κB. It can be assumed that bioaccumulation of AGEs disrupts transcription of genes involved in osteoclastogenesis; consequently, bone remodeling becomes unbalanced due to low bone turnover. This can ultimately lead to reduced bone strength, which could result in increased risk of bone fracture in patients with diabetes.

Disclosure page

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Authors' role

Study concept: KT, KY, SK, YO and YT. Study conduct and data collection: KT, KY, KS and TM. Data analysis and interpretation: KT, KY, SK, SN, YO, SY and YT. All authors participated in the writing of the first draft of the manuscript, and read and approved the final version of the manuscript.

References

[1] I.M. Stratton, A.I. Adler, H.A. Neil, D.R. Matthews, S.E. Manley, C.A. Cull, D. Hadden, R.C. Turner, R.R. Holman, Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study, BMJ 321 (2000) 405–412.
[2] A.V. Schwartz, Diabetes mellitus: does it affect bone? Calcif. Tissue Int. 73 (2003) 515–519.
[3] P. Vestergaard, Discrepancies in bone mineral density and fracture risk in patients with type 1 and type 2 diabetes–a meta-analysis, Osteoporos. Int. 18 (2007) 427–444.
[4] J. Starup-Linde, S.A. Eriksen, S. Lykkeboe, A. Handberg, P. Vestergaard, Biochemical markers of bone turnover in diabetes patients—a meta-analysis, and a methodological study on the effects of glucose on bone markers, Osteoporos. Int. 25 (2014) 1697–1708.
[5] K. Hygum, J. Starup-Linde, T. Harsløf, P. Vestergaard, B.L. Langdahl, MECHANISMS IN ENDOCRINOLOGY: diabetes mellitus, a state of low bone turnover - a systematic review and meta-analysis, Eur. J. Endocrinol. 176 (2017) R137–R157.
[6] R. Singh, A. Barden, T. Mori, L. Beilin, Advanced glycation end-products: a review, Diabetologia 44 (2001) 129–146.
[7] N. Tanaka, H. Yonekura, S. Yamagishi, H. Fujimori, Y. Yamamoto, H. Yamamoto, The receptor for advanced glycation end products is induced by the glycation products themselves and tumor necrosis factor-alpha through nuclear factor-kappa B, and by 17beta-estradiol through Sp-1 in human vascular endothelial cells, J. Biol. Chem. 275 (2000) 25781–25790.
[8] G. Fritz, RAGE: a single receptor fits multiple ligands, Trends Biochem. Sci. 36 (2011) 625–632.
[9] M. Saito, K. Fujii, Y. Mori, K. Marumo, Role of collagen enzymatic and glycation induced cross-links as a determinant of bone quality in spontaneously diabetic WBN/Kob rats, Osteoporos. Int. 17 (2006) 1514–1523.
[10] M. Yamamoto, T. Yamaguchi, M. Yamauchi, S. Yano, T. Sugimoto, Serum pentosidine levels are positively associated with the presence of vertebral fractures in postmenopausal women with type 2 diabetes, J. Clin. Endocrinol. Metab. 93 (2008) 1013–1019.
[11] E. Weinberg, T. Maymon, M. Weinreb, AGEs induce caspase-mediated apoptosis of rat BMSCs via TNFα production and oxidative stress, J. Mol. Endocrinol. 52 (2014) 67–76.
[12] M. Notsu, T. Yamaguchi, K. Okazaki, K. Tanaka, N. Ogawa, I. Kanazawa, T. Sugimoto, Advanced glycation end product 3 (AGE3) suppresses the mineralization of mouse stromal ST2 cells and human mesenchymal stem cells by increasing TGF-β expression and secretion, Endocrinology 155 (2014) 2402–2410.
[13] T. Miyata, K. Notoya, K. Yoshida, K. Horie, K. Maeda, K. Kurokawa, S. Taketomi, Advanced glycation end products enhance osteoclast-induced bone resorption in cultured mouse unfractionated bone cells and in rats implanted subcutaneously with devitalized bone particles, J. Am. Soc. Nephrol. 8 (1997) 260–270.
[14] X.N. Dong, A. Qin, J. Xu, X. Wang, In situ accumulation of advanced glycation endproducts (AGEs) in bone matrix and its correlation with osteoclastic bone resorption, Bone 49 (2011) 174–183.
[15] U. Valcourt, B. Merle, E. Gineyts, S. Viguet-Carrin, P.D. Delmas, P. Garnero, Non-enzymatic glycation of bone collagen modifies osteoclastic activity and differentiation, J. Biol. Chem. 282 (2007) 5691–5703.
[16] Li Z, Li C, Zhou Y, Chen W, Luo G, Zhang Z, Wang H, Zhang Y, Xu D, Sheng P3, Advanced glycation end products biphasically modulate bone resorption in osteoclast-like cells, Am. J. Physiol. Endocrinol. Metab.. 310 (2016) E355–66.
[17] K. Hanami, K. Nakano, K. Saito, Y. Okada, K. Yamaoka, S. Kubo, M. Kondo, Y. Tanaka, Dopamine D2-like receptor signaling suppresses human osteoclastogenesis, Bone 56 (2013) 1–8.
[18] M. Kondo, K. Yamaoka, K. Sakata, K. Sonomoto, L. Lin, K. Nakano, Y. Tanaka, Contribution of the interleukin-6/STAT-3 signaling pathway to chondrogenic differentiation of human mesenchymal stem cells, Arthritis Rheum. 67 (2015) 1250–1260.
[19] X. Zhang, K. Yamaoka, K. Sonomoto, H. Kaneko, M. Satake, Y. Yamamoto, M. Kondo, J. Zhao, I. Miyagawa, K. Yamagata, S. Fukuyo, Y. Okada, Y. Tanaka, Local delivery of mesenchymal stem cells with poly-lactic-co-glycolic acid nanofiber scaffold suppress arthritis in rats, PLoS One 9 (2014) e114621.
[20] K. Yamagata, X. Li, S. Ikegaki, C. Oneyama, M. Okada, M. Nishita, Y. Minami, Dissection of Wnt5a-Ror2 signaling leading to matrix metalloproteinase (MMP-13) expression, J. Biol. Chem. 287 (2012) 1588–1599.
[21] N. Nakano, K. Fukuhara-Takaki, T. Jono, K. Nakajou, N. Eto, S. Horiuchi, M. Takeya, R. Nagai, Association of advanced glycation end products with A549 cells, a human pulmonary epithelial cell line, is mediated by a receptor distinct from the scavenger receptor family and RAGE, J. Biochem. 139 (2006) 821–829.
[22] A.M. Schmidt, S.D. Yan, S.F. Yan, D.M. Stern, The biology of the receptor for advanced glycation end products and its ligands, Biochim. Biophys. Acta 1498 (2000) 99–111.
[23] M. Takeuchi, S. Yamagishi, Involvement of toxic AGEs (TAGE) in the pathogenesis of diabetic vascular complications and Alzheimer's disease, J. Alzheimers Dis. 16 (2009) 845–858.
[24] K. Ohashi, H.K. Takahashi, S. Mori, K. Liu, H. Wake, H. Sadamori, H. Matsuda, T. Yagi, T. Yoshino, M. Nishibori, N. Tanaka, Advanced glycation end products enhance monocyte activation during human mixed lymphocyte reaction, Clin. Immunol. 134 (2010) 345–353.
[25] J.L. Figarola, N. Shanmugam, R. Natarajan, S. Rahbar, Anti-inflammatory effects of the advanced glycation end product inhibitor LR-90 in human monocytes, Diabetes 56 (2007) 647–655.
[26] K. Okazaki, T. Yamaguchi, K. Tanaka, M. Notsu, N. Ogawa, S. Yano, T. Sugimoto, Advanced glycation end products (AGEs), but not high glucose, inhibit the osteoblastic differentiation of mouse stromal ST2 cells through the suppression of osterix expression, and inhibit cell growth and increasing cell apoptosis, Calcif. Tissue Int. 91 (2012) 286–296.
[27] H.K. Takahashi, S. Mori, H. Wake, K. Liu, T. Yoshino, K. Ohashi, N. Tanaka, K. Shikata, H. Makino, M. Nishibori, Advanced glycation end products subspecies-selectively induce adhesion molecule expression and cytokine production in human peripheral blood mononuclear cells, J. Pharmacol. Exp. Ther. 330 (2009) 89–98.
[28] T. Okamoto, S. Yamagishi, Y. Inagaki, S. Amano, K. Koga, R. Abe, M. Takeuchi, S. Ohno, A. Yoshimura, Z. Makita, Angiogenesis induced by advanced glycation end products and its prevention by cerivastatin, FASEB J. 16 (2002) 1928–1930.
[29] Z. Zhou, Y. Tang, X. Jin, C. Chen, Y. Lu, L. Liu, C. Shen, Metformin inhibits advanced glycation end products-induced inflammatory response in murine macrophages partly through AMPK activation and RAGE/NFκB pathway suppression, J. Diabetes Res. 2016 (2016) 4847812.
[30] X. Jin, L. Liu, Y. Zhang, Y. Xiang, G. Yin, Y. Lu, L. Shi, J. Dong, C. Shen, Advanced glycation end products enhance murine monocyte proliferation in bone marrow and prime them into an inflammatory phenotype through MAPK signaling, J. Diabetes Res. 2018 (2018) 2527406.
[31] M. Pertyńska-Marczewska, S. Kiriakidis, R. Wait, J. Beech, M. Feldmann, E.M. Paleolog, Advanced glycation end products upregulate angiogenic and pro-inflammatory cytokine production in human monocyte/macrophages, Cytokine 28 (2004) 35–47.
[32] N. Tahara, S. Yamagishi, M. Takeuchi, A. Honda, A. Tahara, Y. Nitta, N. Kodama, M. Mizoguchi, H. Kaida, M. Ishibashi, N. Hayabuchi, T. Matsui, T. Imaizumi, Positive association between serum level of glyceraldehyde-derived advanced glycation end products and vascular inflammation evaluated by [(18)F] fluorodeoxyglucose positron emission tomography, Diabetes Care 35 (2012) 2618–2625.
[33] S. Pieralice, F. Vigevano, R. Del Toro, N. Napoli, E. Maddaloni, Lifestyle management of diabetes: implications for the bone-vascular axis, Curr. Diab. Rep. 18 (2018) 84.
[34] B. Thompson, D.A. Towler, Arterial calcification and bone physiology: role of the bone-vascular axis, Nat. Rev. Endocrinol. 8 (2012) 529–543.
[35] K. Yanagisawa, J. Ashihara, S. Obara, N. Wada, M. Takeuchi, Y. Nishino, S. Maeda, Y. Ishibashi, S. Yamagishi, Switching to multiple daily injection therapy with glulisine improves glycaemic control, vascular damage and treatment satisfaction in basal insulin glargine-injected diabetic patients, Diabetes Metab. Res. Rev. 30 (2014) 693–700.
[36] A. Nenna, F. Nappi, S.S. Avtaar Singh, F.W. Sutherland, F. Di Domenico, M. Chello, C. Spadaccio, Pharmacologic approaches against advanced glycation end products (AGEs) in diabetic cardiovascular disease, Res. Cardiovasc. Med. 4 (2015) e26949.
[37] M.M. Roorda, Therapeutic interventions against accumulation of Advanced Glycation End products (AGEs), Glycative Stress Res. 4 (2017) 132–143.