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Original article

Glycated-proteins modulate RANKL-induced osteoclastogenesis in RAW264.7 cells.

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Abstract

***Objective*:** Advanced glycation end products (AGEs), such as N^{ε}-(carboxymethyl)-lysine (CML), pentosidine are elevated in serum of osteoporotic patients, which may possibly be due to osteoclastic bone loss. Therefore, we investigated if glycated proteins have any effect on osteoclast differentiation (osteoclastogenesis).

***Methods*:** The murine monocyte/macrophage RAW264.7 along with receptor activators of 100 ng/mL nuclear factor kappa-B ligands (RANKL) was used as a model for osteoclastogenesis. Glycated proteins were prepared using collagen-I, collagen-II or human serum albumin (HSA), first incubating them with glycating agents (glucose, fructose, glycolaldehyde, and glyceraldehyde) in phosphate buffer and then incubating them at 60 °C for 24 or 40 h depending on the protein used. After glycation, non-reacted glycating agents and phosphate buffer were removed by ultrafiltration and used in *in vitro* osteoclastogenesis. Tartrate-resistant acid phosphatase (TRAP) activity was measured to determine osteoclastogenesis, and cell counting kit-8 (CCK-8) and lactate dehydrogenase (LDH) assay were used to check cytotoxicity and real-time polymerase chain reaction (RT-PCR) was used to check mRNA expression.

***Results*:** Glycated proteins significantly modulated RANKL-induced osteoclastogenesis depending on the protein types; glycated collagen-I and II significantly increased, whereas glycated HSA and CML-HSA decreased. None of the experimental AGEs causes cell death. Even though fructose-derived glycated collagen-I obtained 10 times higher fluorescent AGEs than glucose-derived ones, the influence on osteoclastogenesis was almost the same. CML-HSA also showed a trend to downregulate other tested osteoclastogenic and inflammatory mRNA expressions.

***Conclusion*:** Glycated proteins significantly modulated RANKL-induced osteoclastogenesis.

***KEY WORDS*:** osteoclastogenesis, RANKL (receptor activator of nuclear factor kappa-B ligand), glycated-protein, advanced glycation endproducts (AGEs), RAW264.7 cells.

Introduction

Advanced glycation endproducts (AGEs) in the human body (bone, serum, skin, urine, *etc*.) increase with age, and bone mineral density decreases, resulting in high risk of bone fracture. Bone quality is also an important factor in cases of determining bone strength. It is determined by the native structure of the bone building blocks collagen (20% of bone weight) of bones is hydroxyapatite. Collagen is an organic material that losses its native structure due to reactive oxygen species, glycation stress and carbonyl stress, and oxidative stress. Native collagen structures are crucial for maintaining bone health. CML-HSA, pentosidine are the most abundant AGEs found in osteoporotic patients [1-5].

Bone structure and quality are maintained by a process called bone remodeling, which is composed of bone resorption by osteoclast cells and bone formation by osteoblast cells. Osteoclasts originate from hematopoietic stem cells (HSCs) and osteoblasts originate from mesenchymal stem cells (MSCs). Therefore, the differentiation of these two cell types plays crucial role in bone remodeling and thereby, maintaining bone strength [6,7]. Any alteration which occurs in bone microenvironments is resorbed by the osteoclast cell, and later, osteoblasts rebuild the resorbed portion. The differentiation of osteoclast is initiated by the osteocyte and osteoblast cells, and the differentiation of osteoblast is initiated by osteoclast cells by secreting several cytokines like RANKL, macrophage colony-stimulating factor (M-CSF), bone morphogenetic proteins (BMPs), and transforming growth factor beta (TGFβ) [4,8,9].

Both osteoblasts, and osteoclasts, along with their precursors, possess receptor for AGEs (RAGE) and it

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is a multifunctional receptor which plays a vital role in differentiation. RAGE regulates osteoclastogenesis by binding with extracellular high mobility group box 1 (HMGB1) and activates osteoclastogenic downstream signals, thereby supporting osteoclast cells to mature to full functionality [10]. RAGE when binds with AGEs, it triggers to activate several cytokines such as, tumor necrosis factor alpha (TNFα), Interleukin 1 beta (IL-1β), as well as interleukin 6 (IL-6) production that are inflammatory and osteoclastogenic [11-13]. Therefore, we investigated the effect of glycated proteins in RANKL-induced osteoclastogenesis in RAW264.7 cells to check if they have any effect on the differentiation.

Materials and methods

Cell culture and reagents

Murine monocyte/macrophage RAW264.7 (ATCC® TIB-71TM) cell line was purchased from American Type Culture Collection (ATCC; Manassas, VA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan), penicillin 100 units/mL, streptomycin 100 µg/mL and amphotericin B 25 µg/mL (Gibco, El Paso, TX) at 37 °C under the condition of 5% CO_2 [13, 14]. Passages 3 to 6 were used for all experiments.

Glycated-protein preparation

To prepare glycated-collagen, we used 0.6 mg/mL collagen-I (Nippi, Tokyo, Japan) or collagen-II (Elastin Products Co. Inc., Owensville, MO) along with 0.4 M glucose (Glu) or fructose (Fru) in 0.05 M phosphate buffer (pH 7.4) and incubated at 60 °C for 24 h. In case of heated-collagen, we used milliQ instead of glycating agents (Col-I-Heated and Col-II-Heated) [15].

To prepare glycated-HSA, we used 8 mg/mL HSA along with 33 mM glycolaldehyde (Glycol) or glyceraldehyde (Glycer) in 0.05 M phosphate buffer (pH 7.4) and incubated at 60 °C for 40h [15]. In case of heated-HSA, we used milliQ instead of glycating agents. After that, we removed remaining unreacted glycating agents and phosphate buffers using Amicon ultra-4 10K (Millipore, Darmstadt, Germany) centrifugal devices according to the manufacturer's instruction. Briefly, 4 mL of protein mixture was placed into the centrifugal devices and centrifuged at 7,500 × g for 15 min. The mixture was then washed using sterile milliQ and centrifuged again to collect heated or glycated proteins. Protein amount was measured using DC protein assay (Bio-Rad, Hercules, CA) for collagen and BCA protein assay (Thermo Scientific, Rockford, IL) for HSA. CML-HSA was purchased from CircuLex (Nagoya, Aichi, Japan).

Measurement of fluorescent AGEs in our experimental samples:

Filtrated protein samples 200 µg/mL were used to measure fluorescence intensity. Quinine sulfate solution was used as a reference for calibration of fluorescent materials and fluorescence intensity was measured at 370/440 nm using a Varioscan® Flash (Thermo Scientific, Waltham, MA) microplate reader [15, 16].

In vitro osteoclastogenesis

RAW264.7 cells were seeded in multi-well plates and incubated for 24 h for cell attachment. The media were then changed with αMEM (Gibco) to the previously mentioned concentration of heated collagen, glycated collagen or HSA, 100 ng/mL recombinant mouse RANK Ligand (rmRANKL, R&D Systems, Minneapolis, MN) with FBS and antibiotics [17]. After three days, the medium was renewed. After five days of culture, the cells were observed by multiple assays as mentioned.

TRAP activity

Cells treated with the mentioned proteins were fixed after five days of treatment using a cell fixation buffer (acetone : ethanol = 1 : 1). Following this, the fixed cells were used to measure TRAP activity by TRAP solution kit (Oriental Yeast Co., Tokyo, Japan) according to the manufacturer's instruction. Colorimetric absorbances were taken at 405 nm using a Varioscan® Flash microplate reader [17].

Evaluation of cell viability

Cell viability was evaluated using CCK-8 (Dojindo, Kumamoto, Japan). RAW264.7 cells were seeded on 96-well plates at the mentioned density and were treated as regular experiments, which are described in the Results section. After five days, a 10% volume of CCK-8 solution was added to the culture medium and the cells were incubated for 1 h. Absorbance at 450 nm was then measured as previously described [13, 14].

To check cytotoxic cell death, an LDH assay was performed [14]. RAW264.7 cells were seeded and treated as regular experiments. Afterward, cultured media (50 µL) from each well was collected and used to determine the effect of glycated-proteins on cell cytotoxicity (LDH secretion into media) in RAW264.7 cells. After incubating with assay solution and red color development, colorimetric absorbance was measured at 490 nm.

Isolation of total RNA and RT-PCR

RAW264.7 cells were seeded into 24-well plates at a density of 4×10^4 cells/well and incubated for 24 h. Following this, media were changed and incubated for the mentioned times and conditions. Total RNA was then extracted using the Isogen II reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. 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, Waltham, MA).

RT-PCR was performed with a Thunderbird™ SYBR qPCR mix (Toyobo Co., Osaka, Japan) according to the manufacturer's protocol [14] with gene-specific primers (Invitrogen, Tokyo, Japan). The primers were used are as follows: Matrix metallopeptidase 9 (MMP-9), 5′-CTG GAC AGC CAG ACA CTA AAG-3′ (forward), 5′-CTC GCG GCA AGT CTT CAG AG-3′ (reverse); Cathepsin K (CTSK), 5′-GAA GAA GAC TCA CCA GAA GCA G-3′ (forward), 5′-TCC AGG TTA TGG GCA GAG ATT-3′ (reverse); Glyceraldehyde 3-phosphate dehydrogenase

(GAPDH), 5′-AGG TCG GTG TGA ACG GAT TTG-3′ (forward), 5′-TGT AGA CCA TGT AGT TGA GGT CA-3′ (reverse) [18]; TRAP, 5′-GCG ACC ATT GTT AGC CAC ATA CG-3′ (forward), 5′-CGT TGA TGT CGC ACA GAG GGA T-3′ (reverse); ATPase H^+ Transporting V0 Subunit D2 (Atp6v0d2), 5′-ACG GTG ATG TCA CAG CAG ACG T-3′ (forward), 5′-CCT CTG GAT AGA GCC TGC CGC A-3′ (reverse) [19]; TNFα, 5′-ACC CTC ACA CTC AGA TCA TCT TC-3′ (forward), 5′-TGG TGG TTT GCT ACG ACG T-3′ (reverse); IL-1β, 5′-TGT AAT GAA AGA CGG CAC ACC-3′ (forward), 5′-TCT TCT TTG GGT ATT GCT TGG-3′ (reverse); IL-6, 5′-ACA ACC ACG GCC TTC CCT ACT T-3′ (forward), 5′-CAC GAT TTC CCA GAG AAC ATG TG-3′ (reverse); RAGE, 5′-ACT ACC GAG TCC GAG TCT ACC-3′ (forward), 5′-GTA GCT TCC CTC AGA CAC ACA-3′ (reverse); Inducible nitric oxide synthase (iNOS), 5′-CCA AGC CCT CAC CTA CTT CC-3′ (forward), 5′-CTC TGA GGG CTG ACA CAA GG-3′ (reverse) [14]. GAPDH was used as an internal control.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). All statistical analyses were performed using the Tukey-Kramer test for intergroup comparison in all of the experiments. Differences were considered significant at a significance level of 5%.

Results

Glycated-collagen-I stimulated RANKL-induced osteoclastogenesis in RAW264.7 cells:

RAW264.7 cells were treated with RANKL 100 ng/mL with or without glycated-collagen-I (glucose or fructose derived glycated collagen-I; Col-I-Glu, Col-I-Fru, respectively) for five days, followed by an evaluation of osteoclastogenesis by TRAP activity. Cell cytotoxicity was also evaluated using WST-8 assay and LDH secretion into media. Glycated-collagen-I showed no effect on RANKL-induced osteoclastogenesis in lower concentrations (5~50 μg/mL, ***Fig 1-A***), but did show an effect at higher concentrations (200 μg/mL, ***Fig. 1-B***) significantly stimulated without causing cell death (***Fig. 1-C, D***). We also checked the effect of glycated collagen-I in the absence of RANKL and found no stimulatory effect on TRAP activity in RAW264.7 cells (***Fig. 1-B***).

Fluorescent AGEs produced in glycation models:

Glycation of proteins is reported to produce fluorescent AGEs [15, 16]; therefore, we checked fluorescence intensity of the glycated collagen-I at a concentration of 200 μg/mL as its effective dose. Col-I-Fru showed the highest fluorescence intensity compared to Col-I-Glu and Col-I-Heated (***Fig. 2***). Even though Col-I-Fru and Col-I-Glu showed similar effects on osteoclastogenesis, their fluorescence intensity was vastly different, indicating that the effect of glycated collagen-I derived fluorescent AGEs on TRAP activity may have less of a contribution.

Glycated Collagen-II stimulated RANKL-induced osteoclastogenesis in RAW264.7 cells:

As we found glycated-collagen-I stimulated RANKL-induced osteoclastogenesis in RAW264.7 cells, we also checked the effects of glycated-collagen-II (Col-II-Glu, Col-II-Fru). Both Col-II-Fru and Col-II-Glu 200 μg/mL stimulated RANKL-induced osteoclastogenesis (***Fig. 3-A***) without causing cell death (***Fig. 3-B***)

Effect of glycated-collagen-I and glycated-HSA together on osteoclastogenesis:

We found glycated collagen to stimulate RANKL-induced osteoclastogenesis, and in another study, we found glycolaldehyde and glyceraldehyde derived glycated HSA (HSA-Glycol and HSA-Glycer, respectively) inhibited RANKL-induced osteoclastogenesis in RAW264.7 cells [20]. Therefore, we also checked whether both of the stimulatory and inhibitory glycated proteins could neutralize each other's effects or not. Here we found HSA-Glycol and HSA-Glycer 150 μg/mL to inhibit and Col-I-Glu 200 μg/mL to stimulate as our previous study. However, when both were present together, TRAP activity was induced to a greater extent compared to glycated-HSA+RANKL treatment and decreased compared to Col-I-Glu+RANKL and RANKL alone group (***Fig. 4-A***). That result shows that the glycation of proteins have either stimulatory or inhibitory effects depending on the protein type, and together they can modulate RANKL-induced osteoclastogenesis without causing cell death in RAW264.7 cells (***Fig. 4-B***).

CML-HSA also inhibited RANKL-induced osteoclastogenesis:

CML-HSA, a well-known AGE that is responsible for many pathological conditions and glycolaldehyde is known to produce CML-HSA [21]. Therefore, we also checked the effects of CML-HSA, HSA and HSA-Heated on RANKL-induced osteoclastogenesis. We found CML-HSA 0.5 μg/mL also significantly inhibited TRAP activity compared to RANKL with or without HSA and HSA-Heated without glycating agents (***Fig. 5-A***). CML-HSA had increased cells number compared to RANKL treatment alone (***Fig. 5-B***).

Effects of CML-HSA on osteoclastogenic and inflammatory mRNA expression by RANKL-treated RAW264.7 cells.

CML-HSA inhibited osteoclastogenesis, therefore, we investigated the effect of CML-HSA on osteoclastic mRNA (TRAP, CTSK, MMP9, Atp6v, RAGE) and inflammatory and osteoclastogenic mRNA (TNFα, iNOS, IL-1β, IL-6) expression by RAW264.7 cells under our experimental conditions. CML-HSA showed a trend to downregulate all of the tested mRNA expression (***Fig. 6-A~I***). These data suggested that in osteoclastogenic media, CML-HSA has a trend to downregulate osteoclastogenic and inflammatory effects.

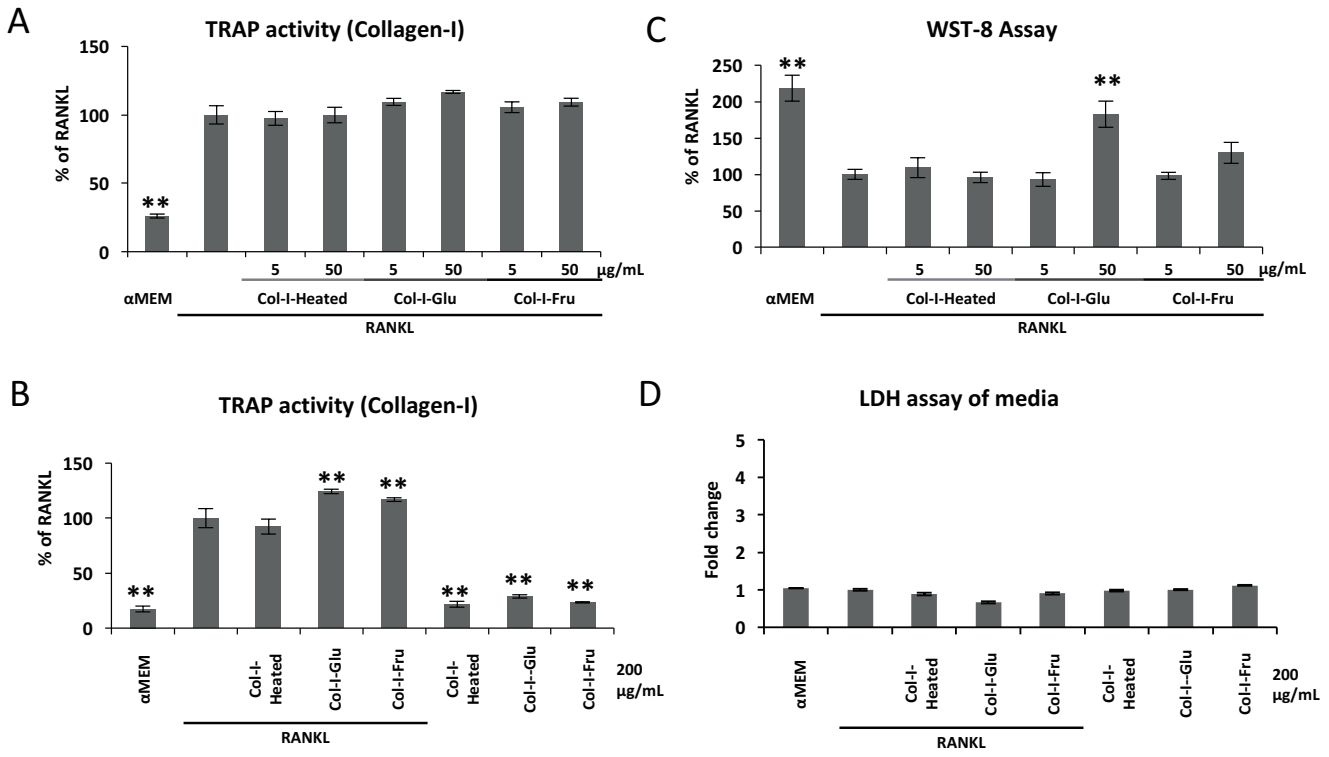

Fig. 1. **Effect of glycated Collagen-I on RANKL-induced osteoclastogenesis.**

RAW264.7 cells were treated with αMEM containing 10% FBS, 100 ng/mL RANKL with or without differing doses of collagen-I (heated, glycated) for 5 days. **A, B.** TRAP activity. **C.** WST-8 assay. **D.** LDH secreted into media. All data are shown as means ± SEM, n = 6. *p < 0.05, **p < 0.01, Tukey-Kramer test. Col-I, collagen type I; Glu, glucose; Fru, fructose; SEM, standard error mean.

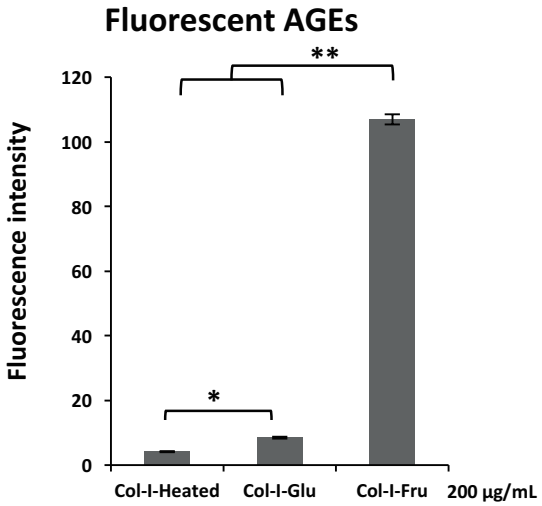

Fig. 2. **Fluorescent AGEs produced in glycation models.**

Fluorescent intensity of glycated collagen-I. All data are shown as means ± SEM, n = 3. *p < 0.05, **p < 0.01, Tukey-Kramer test. AGEs, advance glycation end products; Col-I, collagen type I; Glu, glucose; Fru, fructose; SEM, standard error mean.

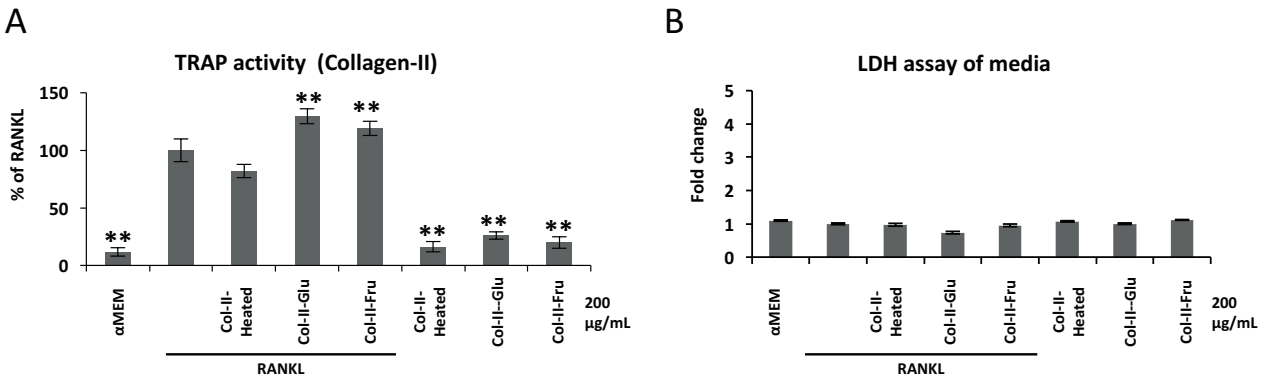

Fig. 3. **Effect of glycated Collagen-II on RANKL-induced osteoclastogenesis.**

RAW264.7 cells were treated with αMEM containing 10% FBS, 100 ng/mL RANKL with or without differing doses of collagen-II (heated, glycated) for 5 days. **A.** TRAP activity. **B.** LDH secreted into media. All data are shown as means ± SEM, n = 6. *p < 0.05, **p < 0.01, Tukey-Kramer test. Coll-II, collagen type II; Glu, glucose; Fru, fructose; SEM, standard error mean.

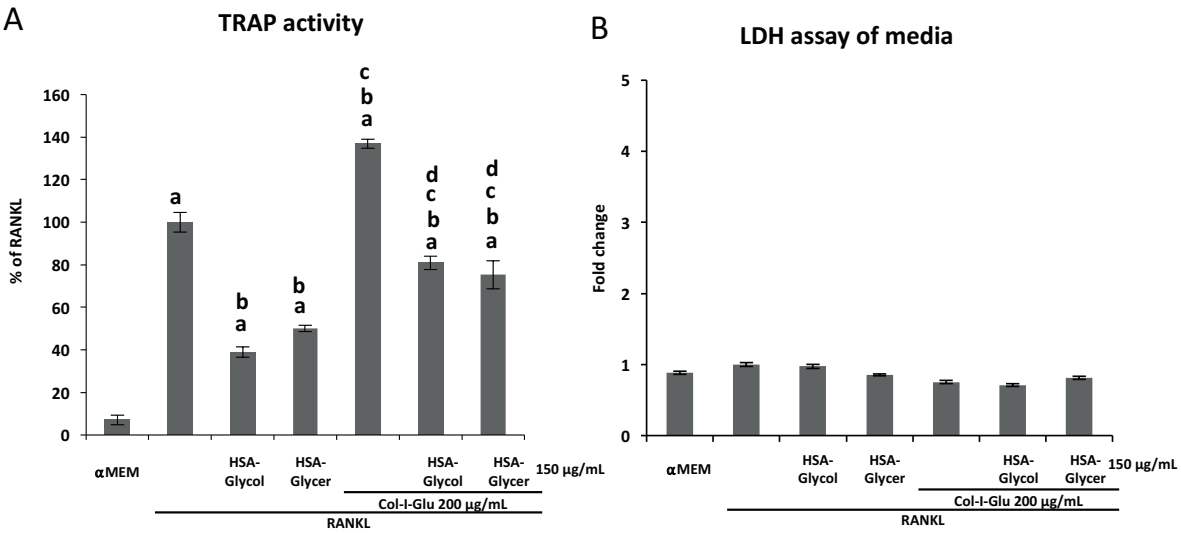

Fig. 4. **Effect of glycated collagen-I (Col-I-Glu) and HSA (HSA-Glycol, HSA-Glycer) together on osteoclastogenesis.**

RAW264.7 cells were treated with RANKL 100 ng/mL along with Col-I-Glu in the presence and absence of HSA-Glycol or HSA-Glycer for 5 days and then TRAP activity was measured **A.** TRAP activity. **B.** LDH assay of media. All data are shown as means ± SEM, n = 6. **a, b, c, d** **p < 0.01 vs αMEM, RANKL, RANKL+Glycated-HSA, RANKL+Glycated-Collagen-I, respectively. Tukey-Kramer test. Col-I, collagen type I; Glu, glucose; Fru, fructose; HSA, human serum albumin; Glycol, glycolaldehyde; Glycer, glyceraldehyde; SEM, standard error mean.

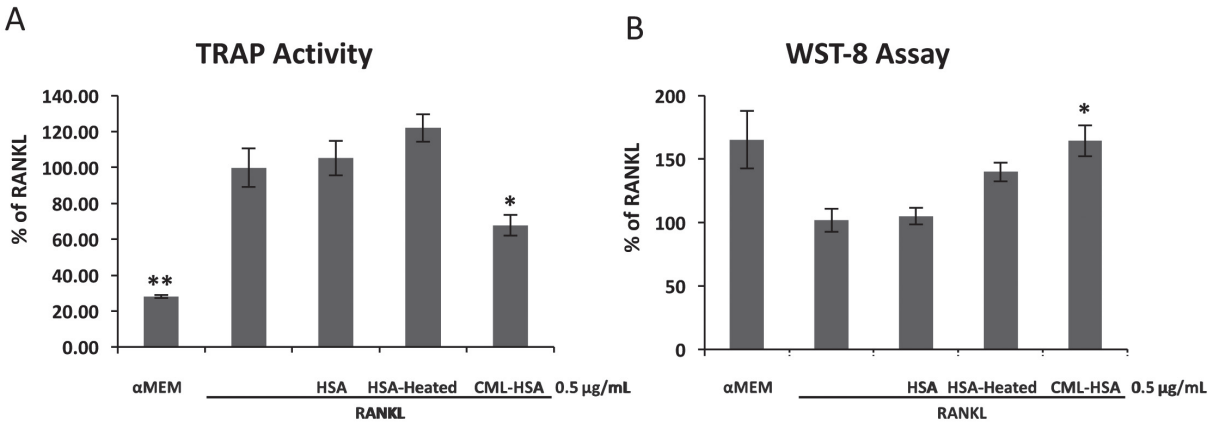

Fig. 5. **Effect of CML-HSA on osteoclastogenesis.**

RAW264.7 cells were treated with αMEM containing 10% FBS, 100 ng/mL RANKL with or without HSA (native), HSA-heated, CML-HSA 0.5 µg/mL for 5 days and then TRAP activity was measured **A.** TRAP activity. **B.** WST-8 assay. All data are shown as means ± SEM, n = 3. *p < 0.05, **p < 0.01, Tukey-Kramer test. CML, N^{ε}-(carboxymethyl)-lysine; HSA, human serum albumin; SEM, standard error mean.

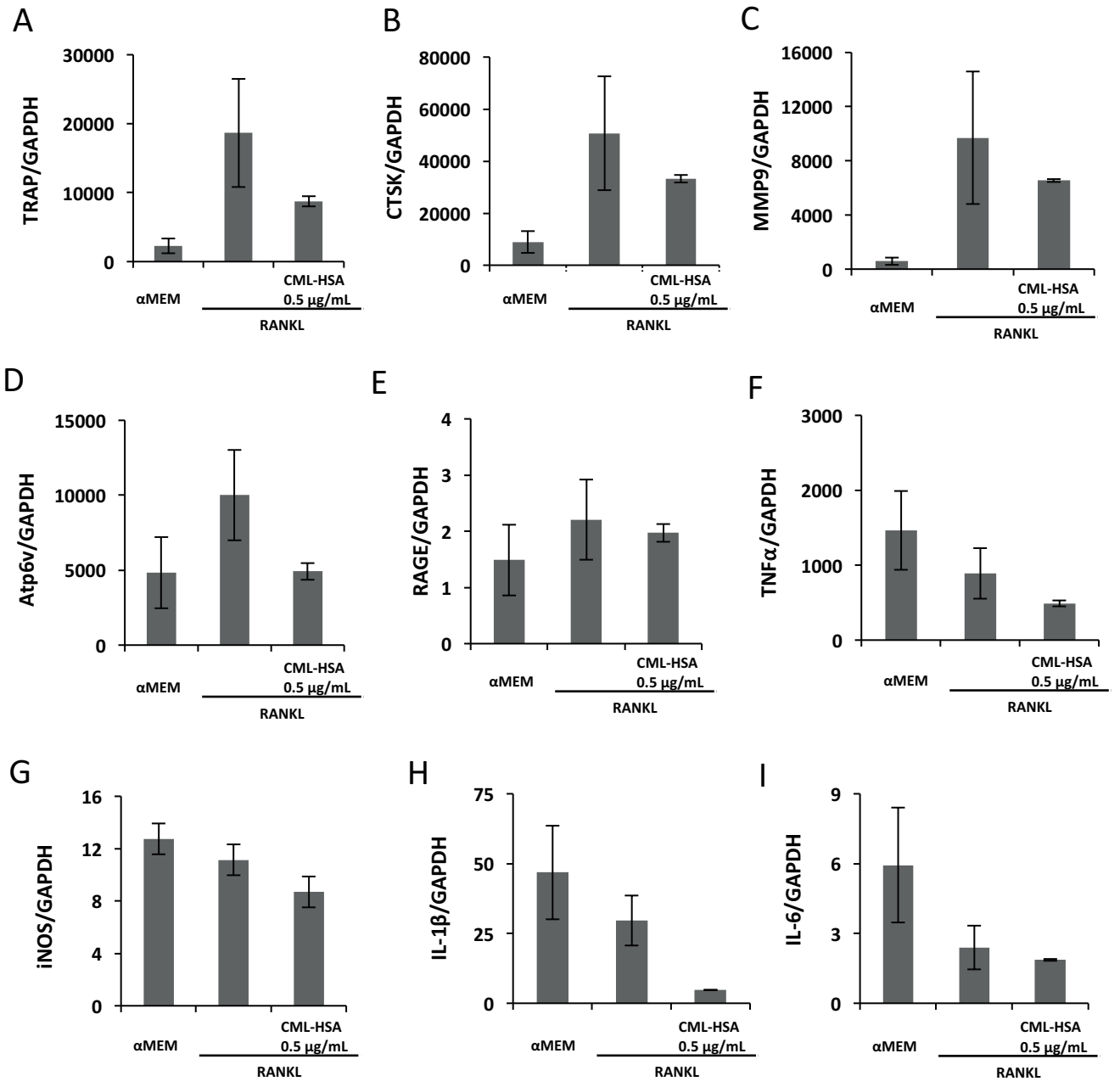

Fig. 6. **Effect of CML-HSA on osteoclastogenic and inflammatory mRNA expression by RANKL-treated RAW264.7 cells.**

RAW264.7 cells were treated with αMEM containing 10% FBS, 100 ng/mL RANKL with or without CML-HSA 0.5 μg/mL for 5 days and then total RNA was used to check mRNA expression using RT-PCR. **A.** TRAP, **B.** CTSK, **C.** MMP9, **D.** Atp6v, **E.** RAGE, **F.** TNFα, **G.** iNOS, **H.** IL-1β, **I.** IL-6. All data are shown as means ± SEM, n = 3. *p < 0.05,**p < 0.01, Tukey-Kramer test. CML, N^{ε}-(carboxymethyl)-lysine; HSA, human serum albumin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CTSK, cathepsin K; MMP, matrix metallopeptidase; Atp6v, ATPase H+ transporting V; RAGE, receptor for AGEs; AGEs, advance glycation end products; TNF, tumor necrosis factor; iNOS, inducible nitric oxide synthase; IL, interleukin; SEM, standard error mean.

Discussion

The CML-HSA and pentosidine levels were reported to be elevated in serum of osteoporosis patients [2-4]. Therefore, we investigated whether glycated protein can modulate osteoclastogenesis or not. We hypothesized either 1) glycated protein is stimulating osteoclastogenesis and thereby increasing bone loss or 2) inhibiting osteoclastogenesis and thereby inhibiting bone remodeling as functional osteoclast cells secrete required cytokines for osteoblastogenesis and therefore bone formation [22-23].

Our study indicated that glycated proteins affected RANKL-induced osteoclastogenesis both positively and negatively depending on the protein types used. Glycated collagen-I (the major organic component of bone matrix) and collagen-II (the major organic component of cartilage) significantly increased RANKL-induced osteoclastogenesis (***Fig. 1-B*** and ***3-A***), whereas glycolaldehyde and glyceraldehyde derived glycated-HSA and CML-HSA significantly inhibited (***Fig. 4-A*** and ***5-A***) without causing cell death (***Fig. 1-D, 3-B, 4-B*** and ***5-B***). Glycated collagen-I (Col-I-Glu) and HSA (HSA-Glycol or HSA-Glycer) together lessened RANKL-induced osteoclastogenesis compared to RANKL with or without Col-I-Glu groups, while it increased than glycated-HSA with RANKL groups (***Fig. 4-A***). This shows that glycated proteins significantly alter the differentiation of RAW264.7 cells into osteoclasts. CML-HSA, a major AGE, which is very common in osteoporotic patients [2-4], also showed an inhibitory effect on osteoclastogenesis (***Fig. 5-A***).

Valcourt *et al*. reported that the resorption of bone and ivory slices in mature rabbit osteoclast cells decreased when they were modified by an AGE (pentosidine). This was likely due to decreased solubility of collagen molecules in the presence of AGEs, whereas AGE-modified bovine serum albumin (BSA) totally inhibited murine and RAW264.7 cells osteoclastogenesis *in vitro* by impairing the commitment of osteoclast ancestors into pre-osteoclast cells [24]. Here, we investigated the effects of glycated collagen-I and II (in soluble form) and HSA on osteoclastogenesis in RAW264.7 cells. We used our *in vitro* model for RANKL-induced osteoclastogenesis in RAW264.7 cells in all of the experiments [17]. Bone resorption usually occurs when the bone microenvironment is altered. In our present study, we found glycation of soluble collagen-I and collagen-II significantly increased RANKL-induced osteoclastogenesis, which shows that glycation of collagen (bone protein) can stimulate osteoclastogenesis. This finding supports our first hypothesis.

In the human body, HSA is present in blood and thereby in bone microenvironments, so we also checked if there is any effect of glycated-HSA on osteoclastogenesis. We observed that HSA-glycol and HSA-glycer are affected negatively by downregulating RANKL-signalling, which supports our second hypothesis [20]. That means, it also can interfere in osteoclastogenesis. Therefore, next, we checked the effect of glycated collagen-I (glucose derived) and HSA-glycol or HSA-glycer together to check if they can counteract each other's effects. Both of the glycated proteins significantly countered each other's effects in our study, which provides evidence for modulation of osteoclastogenesis by glycated proteins.

CML-HSA is reported to produce by glycolaldehyde [21], therefore, we also checked if CML-HSA has such effects on osteoclastogenesis. CML-HSA also significantly inhibited RANKL-induced osteoclastogenesis, showing that glycated protein (AGEs) can alter osteoclastogenesis. We also checked the effect of CML-HSA on osteoclast marker (TRAP, MMP9, CTSK, Atp6v) [8,18], osteoclastogenic and inflammatory cytokines (TNFα, IL-1β, IL-6, *etc*.) [6,12] along with RAGE mRNA expression under our experimental conditions. CML-HSA showed a trend to downregulate tested mRNA expression, indicating that CML-HSA has a trend to downregulate osteoclastogenic and inflammatory effects on RANKL-induced *in vitro* osteoclastogenic model of RAW264.7 cells.

Conclusion

Glycation of proteins can significantly interfere with RANKL-induced osteoclastogenesis in RAW264.7 cells.

Acknowledgements

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Conflict of interest

The authors claim no conflict of interest in this study.

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