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Original Article

Continuous Stimulation with Glycolaldehyde-derived Advanced Glycation End Product Reduces Aggrecan and COL2A1 Production via RAGE in Human OUMS-27 Chondrosarcoma Cells

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Chondrocytes are responsible for the production of extracellular matrix (ECM) components such as collagen type II alpha-1 (COL2A1) and aggrecan, which are loosely distributed in articular cartilage. Chondrocyte dysfunction has been implicated in the pathogenesis of rheumatic diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA). With age, advanced glycation end products (AGEs) accumulate in all tissues and body fluids, including cartilage and synovial fluid, causing and accelerating pathological changes associated with chronic diseases such as OA. Glycolaldehyde-derived AGE (AGE3), which is toxic to a variety of cell types, have a stronger effect on cartilage compared with other AGEs. To understand the long-term effects of AGE3 on cartilage, we stimulated a human chondrosarcoma cell line (OUMS-27), which exhibits a chondrocytic phenotype, with 10 μg/ml AGE3 for 4 weeks. As a result, the expressions of COL2A1 and aggrecan were significantly downregulated in the OUMS-27 cells without inducing cell death, but the expressions of proteases that play an important role in cartilage destruction were not affected. Inhibition of the receptor for advanced glycation end products (RAGE) suppressed the AGE3-induced reduction in cartilage component production, suggesting the involvement of RAGE in the action of AGE3.

Key words: advanced glycation end product, aging, cartilage, collagen, aggrecan

Rheumatic diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA) are degenerative joint conditions that affect millions of people worldwide [1,2]. They lead to a progressive degeneration of articular cartilage that results in joint pain, stiffness, and reduced range of motion [3]. Chondrocytes, the predominant cell type in cartilage tissue, are silent and rarely divide under physiological conditions [4], but in rheumatic diseases chondrocytes act as target cells for inflammatory mediators such as cytokines, leading to chondrocyte dysfunction [5,6]. Chondrocytes are responsible for synthesizing components of the extracellular matrix (ECM), including collagens and aggre-

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can, which provide mechanical support to cartilage during joint motion [7]. An imbalance between the anabolic and catabolic processes in the ECM substantially affects cartilage degradation. Altered synthesis and turnover of ECM components, such as collagen type II alpha-1 (COL2A1) and aggrecan, contribute to loss of cartilage integrity [8, 9]. The degradation of cartilage matrix in OA is mediated by proteases. In OA, COL2A1 is primarily degraded by the matrix metalloproteinases (MMP)1, -3, and -13, whereas aggrecan is primarily degraded by ADAM metallopeptidase with thrombospondin type 1 motif 4 (ADAMTS)4, -5, and -9 [10].

Advanced glycation end products (AGEs) are formed by the non-enzymatic reaction of reducing sugars with proteins, lipids, or nucleic acids [11] with the receptor for AGEs (RAGE) being a major receptor for AGE signaling [12]. Among the AGEs, glycolaldehyde-derived AGE (AGE3), which is toxic to a variety of cell types, plays a role in the pathogenesis of diabetic complications, as it exerts stronger activity on cartilage compared to other AGEs such as glucose-derived AGE (AGE1) [13, 14]. In patients with OA, a strong OA degree-dependent relationship between AGE3 and RAGE has been observed, and AGE3 has been shown to be the most important AGE for cartilage degeneration through the RAGE pathway [15]. Moreover, type 2 diabetes has been reported to contribute to the progression and pathogenesis of OA, including via a mechanism involving AGEs in the dysregulation of articular cartilage metabolism [16].

Accumulation of AGEs is an inevitable part of the aging process in the human body, and recent studies have linked AGEs to age-related disease complications such as cardiovascular diseases and Alzheimer's disease [17]. Limiting the intake of dietary AGEs provides a protective effect under conditions such as wound healing and insulin resistance. An increase in lifespan in animal models following restricted AGE intake has been observed [18]. AGEs have also been suggested to accumulate in articular cartilage with aging, affecting cartilage function [19, 20]. However, the influence of prolonged direct exposure to AGEs on articular cartilage remains unclear.

In the present study, we investigated both the effects of a 4 week exposure to 10 μg/ml AGE3 on the expressions of COL2A1 and aggrecan in a human chondrosarcoma cell line (OUMS-27) and the role of RAGE in mediating these effects.

Methods

Reagents and antibodies. Anti-COL2A1 and anti-aggrecan antibodies (catalogue nos. sc-52658 and sc-166951; Santa Cruz Biotechnology, Santa Cruz, CA, USA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (no. MAB374; Millipore, Bedford, MA, USA), and horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibodies (no. 5220-0341; SeraCare Life Sciences, Milford, MA) were used in this study. AGE3 was prepared as described previously [21, 22]. Briefly, bovine serum albumin (BSA) (Fujifilm Wako, Osaka, Japan) was incubated with glycolaldehyde dimer (Sigma-Aldrich) in 0.2 M phosphate buffer (pH 7.4) at 37°C for 7 days under sterile conditions. The AGEs were then dialyzed for 2 days against phosphate-buffered saline (PBS) at 4°C. The RAGE antagonist FPS-ZM1 was purchased from Millipore.

Cell culture and treatment. We used OUMS-27 cells in this study because primary chondrocytes become unstable and lose their properties when cultured for extended periods *in vitro* [23]. Originally established as a chondrosarcoma cell line, OUMS-27 cells retain chondrocytic differentiation and express key markers such as types I, II, and III collagen, making this cell line a valuable model for studying human chondrocytes and chondrosarcoma [24]. Other research groups have also used OUMS-27 cells as a substitute for primary chondrocytes [25, 26].

The experimental design is illustrated in Fig. 1A, B. We cultured OUMS-27 cells in Dulbecco's modified Eagle medium (DMEM; Nissui Pharmaceutical, Tokyo) supplemented with 10% fetal bovine serum (FBS) and maintained the cultures at 37°C in a humidified chamber with 5% CO_2 and 20% O_2. For the assessment of the long-term effects of AGE3 over an 8-week duration, 5.0×10^5 OUMS-27 cells were seeded in 10 cm culture plates and passaged weekly, with each passage replated into fresh 10 cm plates upon reaching near-confluence. On the passages at the 1st, 3rd, and 7th weeks, cells were also seeded into 6-well plates (1.0×10^5) for protein isolation and 12-well plates (5.0×10^4) for mRNA isolation. After 1 week of culture with or without AGE3, protein and mRNA were isolated at 1, 2, 4, and 8 weeks. For evaluation of the short-term effects of AGE3 over a 24-h period, OUMS-27 cells (1.0×10^5) were seeded

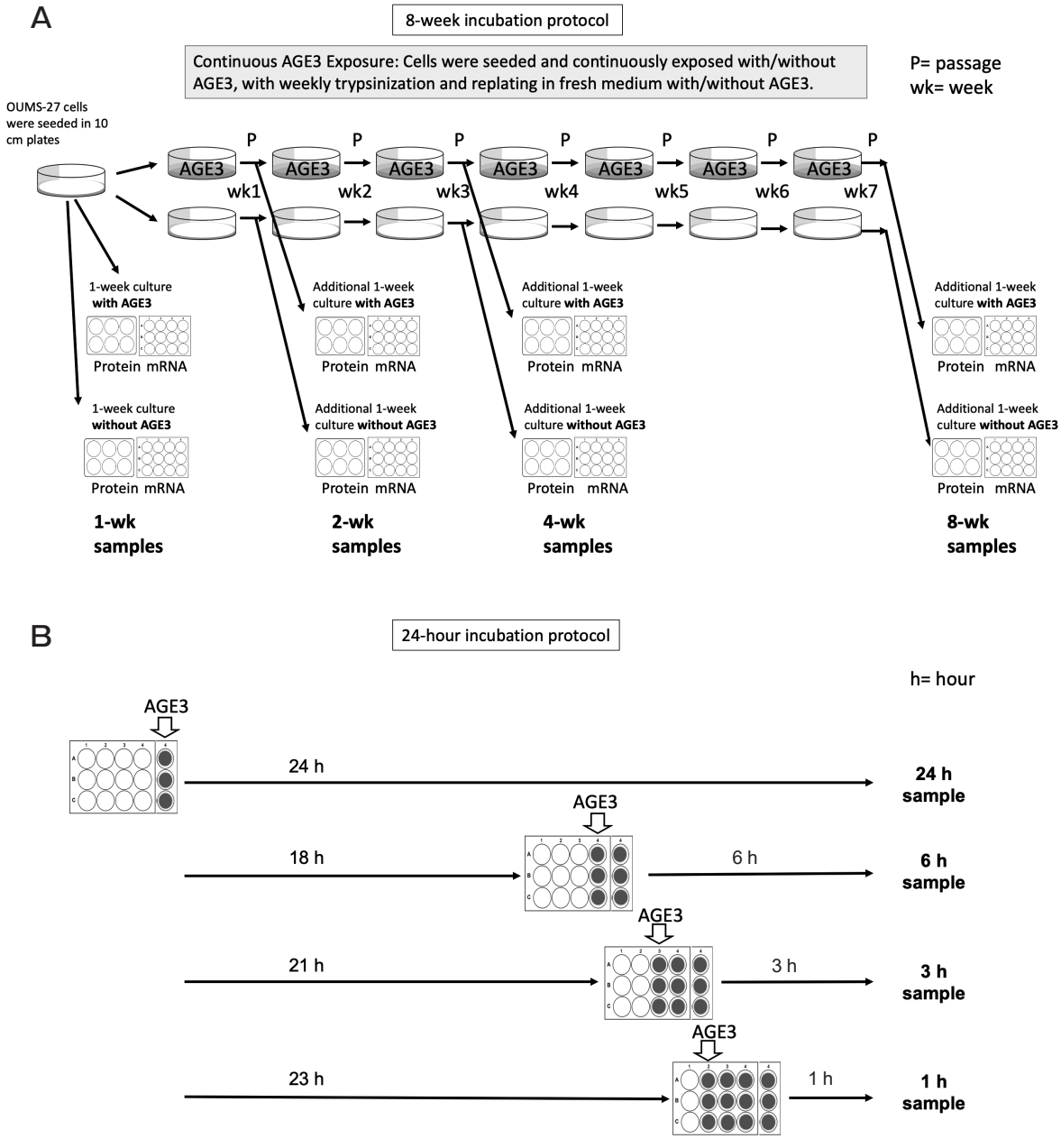

Fig. 1 The experimental design of the study. (A) OUMS-27 cells were cultured in DMEM supplemented with 10% FBS. Cells (5.0×10^5) were initially seeded in 10 cm culture plates and passaged weekly, with each passage replated into fresh 10 cm plates. At each passage, cells were also seeded into 6-well plates (1.0×10^5) for protein isolation and 12-well plates (5.0×10^4) for mRNA isolation. Cells were cultured either in the presence or absence of AGE3. Protein and mRNA were isolated at 1, 2, 4, and 8 weeks after the start of the culture. (B) For the 24-h protocol, cells were seeded into 12-well plates (2.5×10^5) for mRNA isolation. The cells were stimulated with AGE3, and samples were collected at 1, 3, 6, and 24 h after stimulation for mRNA isolation.

into 12-well plates for mRNA isolation. AGE3 was added to the wells, and samples were collected at specific time points. For 24 h samples, AGE3 was added at the start and incubated for 24 h. For 6 h, 3 h, and 1 h samples, AGE3 was added after 18, 21, and 23 h, respectively, with collection at 24 h. At the end of the

24 h period, mRNA was isolated from all samples.

Cell viability assay. The viability of OUMS-27 cells was assessed using a CCK-8 kit (Dojindo, Tokyo). Briefly, OUMS-27 cells were seeded in 96-well plates (5.0×10^5 cells) and exposed to AGE3. Cells were then incubated for 1-8 days at 37°C with 5% CO_2, and the optical density was measured at 450 nm using a microplate reader (model 680; Bio-Rad Laboratories, Hercules, CA, USA). Cell viability was determined as a percentage of proliferation compared with that of control cells.

RNA extraction and quantitative real-time polymerase chain reaction (qPCR). Total RNA was extracted from cell samples with the use of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's guidelines. The RNA concentration was measured at 260 nm using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The extracted total RNA was then subjected to reverse transcription using ReverTra Ace (Toyobo, Osaka, Japan). The reaction was carried out at 25°C for 10 min, 42°C for 60 min, and 97°C for 5 min using random primers (Toyobo).

The qPCR was performed on a StepOnePlus system (Applied Biosystems, Foster City, CA, USA) using SYBR Green PCR Master Mix (Applied Biosystems). The PCR reaction mixture consisted of 5 µl of 2× SYBR Green mix and 0.5 µl (10 µM) of each target gene primer, using GAPDH as an endogenous internal control gene. The reaction was performed in a 96-well reaction plate under the following conditions: 10 min at 95°C, followed by 40 cycles of 3 s at 95°C and 30 s at 60°C. The mRNA expression levels of target genes were determined using the comparative Cq (ΔΔCq) method and normalized to GAPDH expression. The sequences of the primers used for the qPCR are listed in Table 1.

Western blot analysis. OUMS-27 cells at a density of 1.0×10^6 cells/well were cultured in 6-well plates using DMEM supplemented with 10% FBS at 37°C and 5% CO_2. Following AGE3 stimulation, the cells were washed with cold PBS and lysed in radio-immunoprecipitation assay (RIPA) buffer containing protease inhibitors for protein extraction as described previously [27]. The inhibitors used were antipain, leupeptin, and aprotinin at a concentration of 2.0 µg/ml each (Peptide Institute, Osaka, Japan; Nacalai Tesque, Kyoto, Japan). After centrifugation, the total protein concentration was determined using a Bradford protein assay kit (Bio-Rad Laboratories). The cell lysates were then mixed with 6× reducing sample buffer, denatured at 95°C for 5 min, and separated on 8% SDS-PAGE gels. The separated proteins were then transferred to polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany).

The primary antibodies used in the experiment were anti-COL2A1 and anti-aggrecan at a dilution of 1 : 1,000 and anti-β-actin at 1 : 10, 000 (used as control). Anti-mouse IgG secondary antibodies were used at dilutions of 1 : 2,000 and 1 : 10,000, respectively. The membranes were blocked with 5% skim milk in TBS-0.05% Tween 20 (TBS-T) for 1 h at 25°C (room temperature), followed by incubation with primary antibodies overnight at 4°C. After three washes with 1×

Table 1 Sequence of qPCR primers

Gene name	Forward (5'–3')	Rewerse (5'–3')	Product size (bp)
MMP1	ATGAAGCAGCCCAGATGTGGAG	TGGTCCACATCTGCTCTTGGCA	137
MMP2	AGCGAGTGGATGCCGCCTTTAA	CATTCCAGGCATCTGCGATGAG	138
MMP3	CACTCACAGACCTGACTCGGTT	AAGCAGGATCACAGTTGGCTGG	156
MMP9	TTCCAAACCTTTGAGGGCGA	CAAAGGCGTCGTCAATCACC	106
MMP13	CCTTGATGCCATTACCAGTCTCC	AAACAGCTCCGCATCAACCTGC	121
ADAMTS4	CACTGACTTCCTGGACAATGGC	GGTCAGCATCATAGTCCTTGCC	104
ADAMTS9	AGGACAAGCGAAGGACATCC	CATCCGGCGACTTCTTGATG	181
COL2A1	CGTCCAGATGACCTTCCTACG	TGAGCAGGGCCTTCTTGAG	122
AGGRECAN	AGGCAGCGTGATCCTTACC	GGCCTCTCCAGTCTCATTCTC	136
RAGE	CACCTTCTCCTGTAGCTTCAGC	GGAGCTACTGCTCCACCTTC	141
TLR4	TCCTGCGTGAGACCAGAAAG	GGGAGGTTGTCGGGGATTTT	104
FEEL1	GAACCATGTGCCACTGGAAGG	AGCGGAATCTCCTGGTGCAGT	155
LOX1	ACCTTCCCCTCACACTCCTA	TTTCGGAATGGCCTCTGTCC	180
GAPDH	CCACAGTCCATGCCATCACT	GGCAGGGATGATGTTCTGGAG	101

TBS-T buffer, the membranes were incubated with secondary antibodies for 1 h at 25°C. The chemiluminescent signals were visualized using Amersham™ ECL™ Prime (Cytiva, Marlborough, MA, USA), imaged using an Amersham Imager 600, and densitometrically quantified using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA).

Statistical analyses. The resulting data are expressed as the mean ± SEM (standard error of the mean). Differences between groups were analyzed by a one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* comparison. All experiments were repeated at least three times. In all analyses, a p-value < 0.05 was considered significant.

Results

The expressions of COL2A1 and aggrecan in OUMS-27 were reduced by 4-week AGE3 stimulation. We investigated both short- and long-term effects of AGE3 on the expression levels of *COL2A1* and *aggrecan* in the chondrosarcoma cell line OUMS-27 following treatment with different concentrations of AGE3 (5, 10, 20, and 50 μg/ml) for different durations (1, 3, 6, and 24 h and 1, 2, 4, and 8 weeks). We found that the expression levels of *COL2A1* and *aggrecan* were significantly decreased after 4 weeks of AGE3 stimulation compared to the levels in the control group (Fig. 2A, B). However, incubation for 1, 3, 6, and 24 h with AGE3 (10 μg/ml) did not affect the expression level of *COL2A1* or *aggrecan* in this human cell line (Fig. 2C).

We then performed western blotting to measure the expression levels of COL2A1 and aggrecan proteins in OUMS-27 cells treated for 4 weeks with different concentrations of AGE3 (0, 5, 10, 20, and 50 μg/ml). AGE3 stimulation significantly decreased the expression levels of COL2A1 and aggrecan proteins compared to the levels in the control group (Fig. 2D-F).

The RAGE-specific inhibitor FPS-ZM1 attenuated AGE-induced degradation of COL2A1 and aggrecan. Both AGEs and their receptors are considered to play important roles in AGE-related complications. We verified the expressions of *RAGE*, *TLR4*, *FEEL1*, and *LOX1*, which are known AGE-related receptors [28], in OUMS-27 cells (data not shown), and we then investigated whether their expressions were affected by AGE stimulation. The quantitative real-time PCR analysis demonstrated that stimulation with 10 μg/ml AGE3 for 2 or 4 weeks significantly upregulated the expression of *RAGE*, whereas the expressions of *TLR4*, *FEEL1*, and *LOX1* remained unaffected (Fig. 3A). Based on these findings, we investigated the possibility of reversing the AGE3-induced decrease in COL2A1 and aggrecan expressions in OUMS-27 cells by using a small molecule inhibitor of RAGE, *i.e.*, FPS-ZM1. Notably, pretreatment with 1 μM FPS-ZM1 significantly attenuated all AGE3-mediated effects on the expressions of COL2A1 and aggrecan (Fig. 3B-D). These results indicated the involvement of RAGE in the action of AGE3.

Protease expression in OUMS-27 cells was not affected by AGE3 treatment. Proteases are involved in the degradation of cartilage matrix in OA. Cartilage matrix degradation is driven by the activity of catabolic enzymes and inflammatory mediators, including MMPs and ADAMTSs [29, 30]. We thus investigated the mRNA expression levels of MMP1, MMP2, MMP3, MMP9, MMP13, ADAMTS4, and ADAMTS9 in response to 10 μg/ml AGE3 treatment for various durations (1, 3, 6, and 24 h and 1, 2, 4, and 8 weeks) to evaluate the effect of AGE3 on these expressions. The treatment with 10 μg/ml AGE3 had no effect on the expressions of these catabolic enzymes at 1, 3, 6, and 24 h (Fig. 4A) or at 1, 2, 4, and 8 weeks (Fig. 4B).

Evaluation of the time- and dose-dependent cytotoxicity of AGE3 on OUMS-27 cells by CCK-8 assay. To investigate the effect of AGE3 on the viability of OUMS-27 cells, we incubated cells with different concentrations of AGE3 (10, 20, 50, 100, and 200 μg/ml) for various durations (1, 2, 4, and 8 days) and evaluated cell viability by conducting a CCK-8 assay. AGE3 concentrations below 50 μg/ml did not have a significant effect on the cell proliferation, as shown in Fig. 5. This observation suggested that OUMS-27 cells are able to maintain resistance to AGE-induced toxicity by preserving their viability in the presence of AGE3 at the concentrations used in this study.

Discussion

AGEs accumulate in articular cartilage over time, impairing cartilage function and leading to arthritis; this accumulation of AGEs causes cartilage browning, as revealed in fluorescence microscopy [31]. The mechanism of action of AGEs involves crosslinking with joint proteins — including collagen and aggrecan — and RAGE binding, which activates a number of intracellular

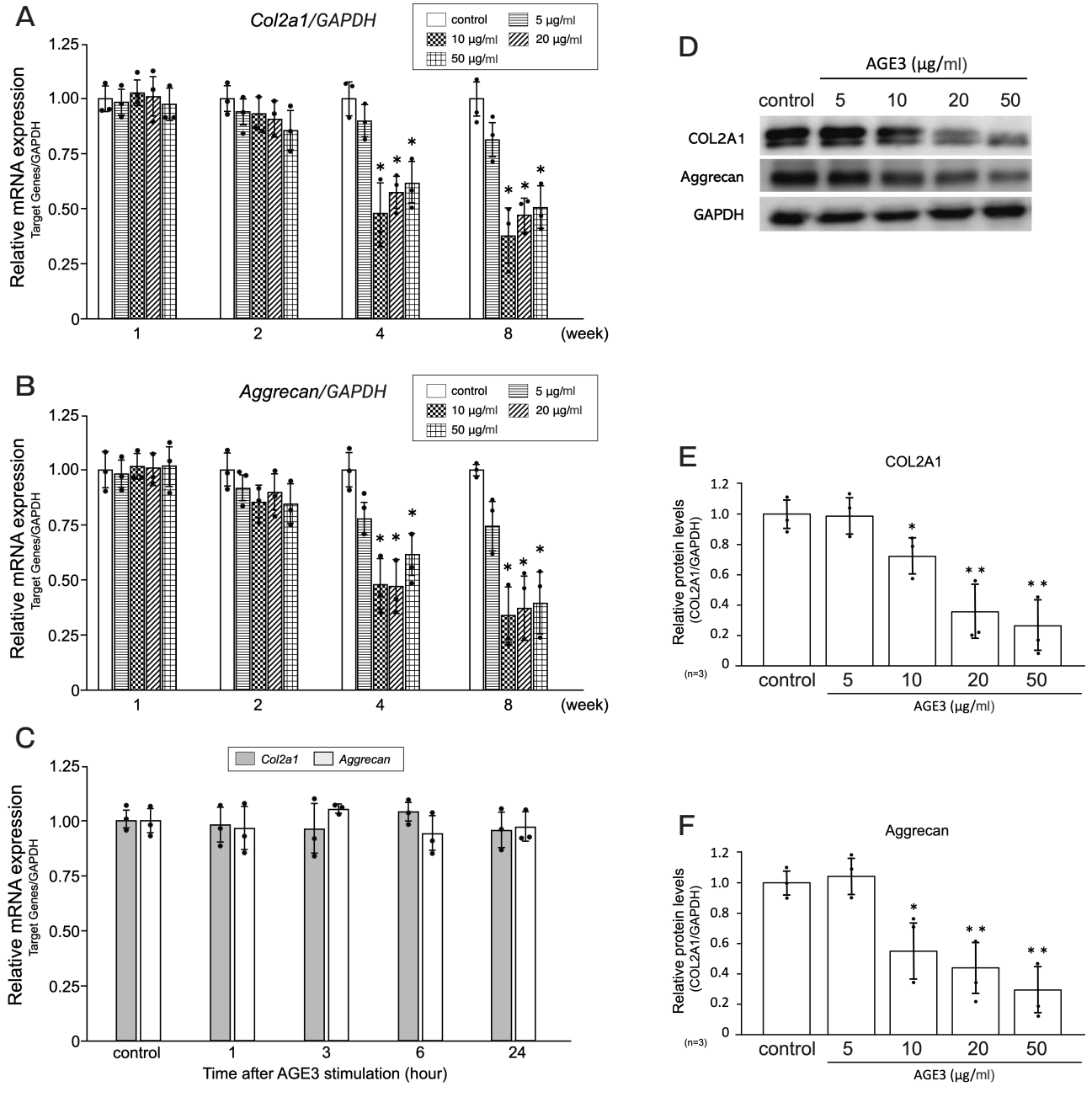

Fig. 2 AGE3 induced reductions in the expressions of COL2A1 and aggrecan in OUMS-27 cells over a 4-week period. The *COL2A1* and *aggrecan* expressions in long-term AGE3-stimulated OUMS-27 cells were assessed by a quantitative real-time PCR. The continuous stimulation of OUMS-27 cells with AGE3 decreased the *COL2A1* and *aggrecan* expressions after 4 weeks. (**A**) *COL2A1* and (**B**) *aggrecan* expression. (**C**) AGE3 Treatment (10 $\mu g/ml$) for 1-24 h. Short-term incubation (1, 3, 6, and 24 h) with 10 $\mu g/ml$ AGE3 had no effect on *COL2A1* or *aggrecan* expression. (**D**) The western blot analysis revealed a significant AGE3-induced downregulation of COL2A1 and aggrecan expression in OUMS-27 cells after 4 weeks compared to that in the controls. GAPDH was used as an internal control. (**E**) The ratio COL2A1 and (**F**) aggrecan protein expressions were measured using ImageJ software. Each bar represents the mean ± SEM of three independent experiments. Data were analyzed by a one-way ANOVA followed by Dunnett's *post hoc* test. $*p<0.05$, $**p<0.01$ vs. control.

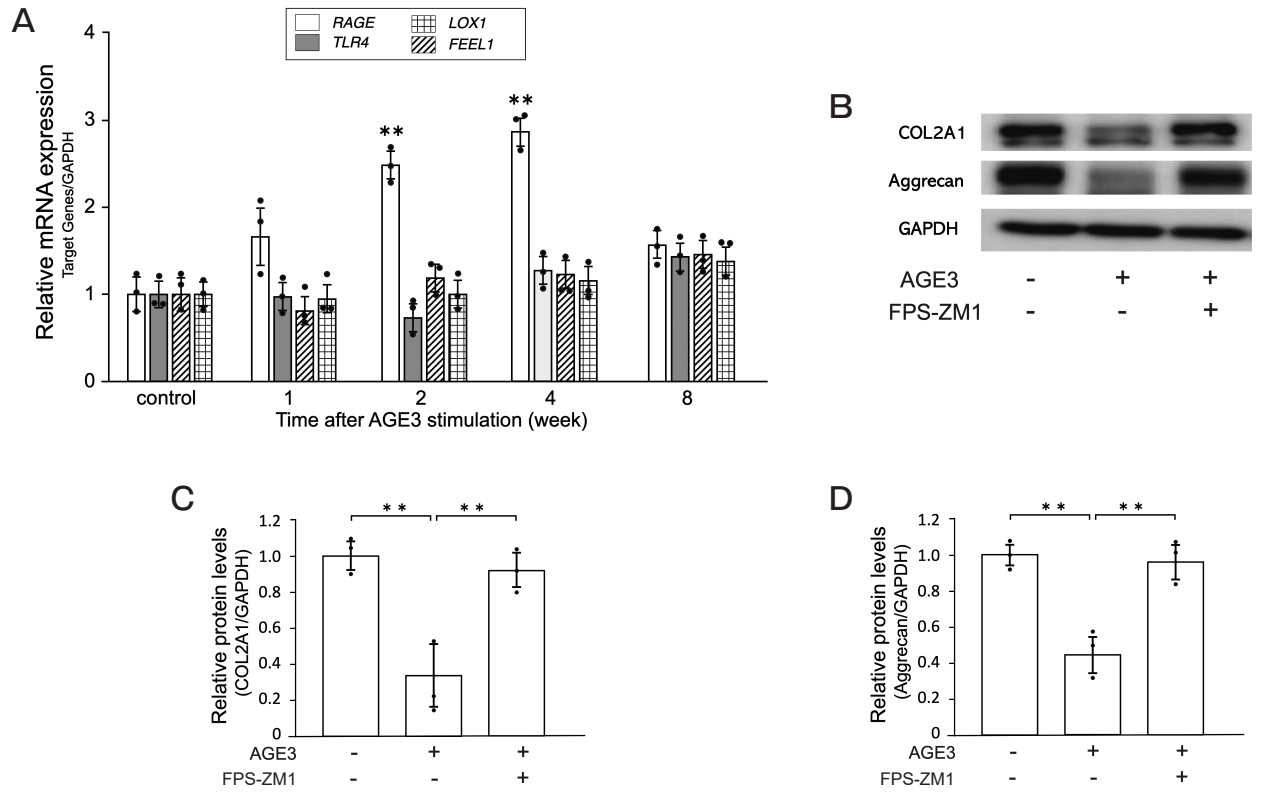

Fig. 3 A RAGE-specific inhibitor reduced AGE-induced COL2A1 and aggrecan degradation. AGE3 stimulation significantly upregulated the expression of *RAGE*, whereas treatment with the RAGE-specific inhibitor FPS-ZM1 attenuated the AGE-induced decrease in COL2A1 and aggrecan expressions in OUMS-27 cells. (**A**) AGE3 Treatment (10 μg/ml) for 1-8 weeks. Stimulation with 10 μg/ml AGE3 significantly increased the expression of *RAGE* after 2 weeks while not affecting that of *TLR4*, *FEEL1*, or *LOX1*. (**B**) Western blot analysis revealed that pretreatment with 1 μM FPS-ZM1 significantly reduced all 4-week AGE3-mediated effects on the COL2A1 and aggrecan expressions in OUMS-27 cells compared to those in the controls. GAPDH was used as an internal control. (**C**) The ratio COL2A1 and (**D**) aggrecan protein expressions were measured with ImageJ software. Bars: mean ± SEM of three independent experiments. A one-way ANOVA followed by Dunnett's or Tukey's *post hoc* test was used. $^{**}p<0.01$. vs. control.

signaling pathways [32]. This supports the observations that AGE accumulation plays a pivotal role in cartilage dysfunction and is directly linked to arthritis progression through its interaction with essential cartilage components and receptor-mediated pathways.

The present study revealed for the first time that 4 weeks of stimulation with 10 μg/ml AGE3 in a human chondrocyte-like cell line led to a decrease in the expressions of COL2A1 and aggrecan via RAGE without affecting chondrocyte viability, whereas stimulation with 10 μg/ml AGE3 treatment for 1, 3, 6, or 24 h did not affect the expression level of COL2A1 or aggrecan in the cells (Fig. 2). These findings are critical in demonstrating the dramatic catabolic effect of low doses of AGEs, which better replicate the biological environment than high-level AGE doses on aggrecan and COL2A1 after prolonged stimulation. Collagen and aggrecan are key components of the cartilage matrix and are essential for the maintenance of normal cartilage cells. Both proteins decline with age, and their dysfunction accelerates the progression of rheumatic diseases such as OA and RA [1, 33]. Inhibiting the loss of collagen and aggrecan in rheumatic diseases may therefore have the potential to prevent cartilage degeneration. This suggests that sustained low-level AGE exposure over time may have significant implications in the degradation of cartilage matrix components, even at concentrations resembling physiological conditions.

In vitro studies have shown that treatment with 100 μg/ml AGEs for 24 h reduces the expressions of

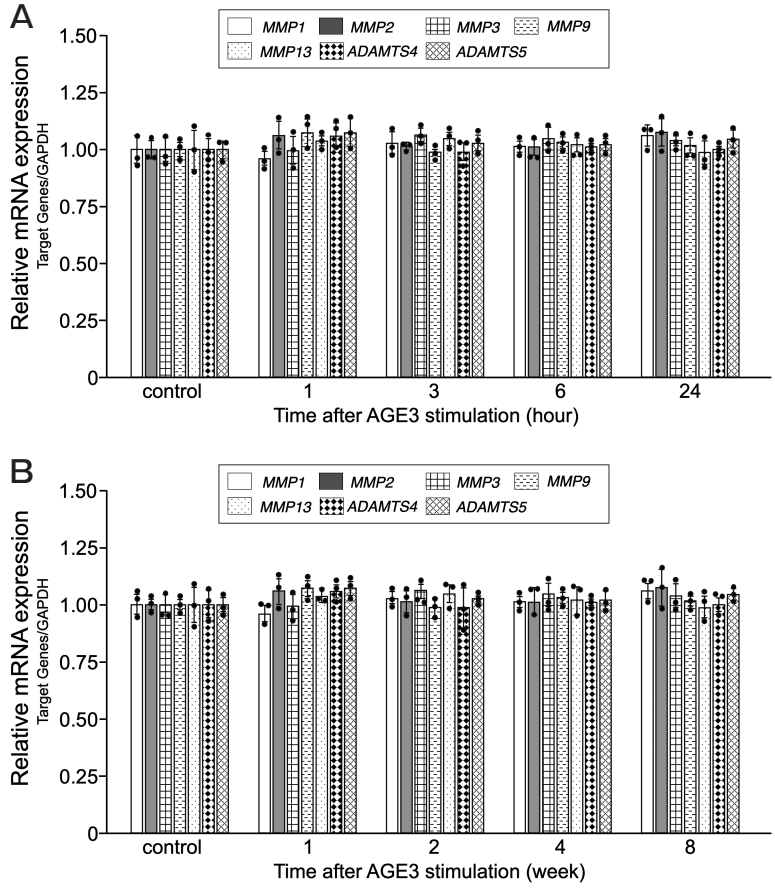

Fig. 4 AGE3 treatment in OUMS-27 cells did not affect the protease expression. The time course of the expressions of *MMP1*, *MMP2*, *MMP3*, *MMP9*, *MMP13*, *ADAMTS4*, and *ADAMTS*9 in AGE3-stimulated OUMS-27 cells is shown. (**A**) AGE3 Treatment (10 μg/ml) for 1-24 h. Stimulation with 10 μg/ml AGE3 for up to 24 h did not affect the expressions of target genes. (**B**) AGE3 Treatment (10 μg/ml) for 1-8 weeks. Stimulation with 10 μg/ml AGE3 for up to 8 weeks did not affect the expression of target genes. Bars: mean $\pm$ SEM of three independent experiments. A one-way ANOVA followed by Dunnett's *post hoc* test was used.

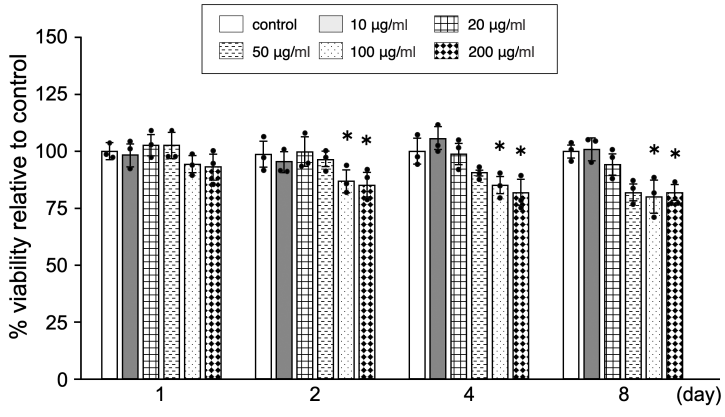

Fig. 5 Determination of AGE3 cytotoxicity on OUMS-27 cells. Incubation with different concentrations of AGE3 (10, 20, 50, 100, and 200 μg/ml) for different durations (1, 2, 4, and 8 days) was conducted. The low concentrations of AGE3 ($\leq$50 μg/ml) had no significant effect on the proliferation of cells. Bars: mean $\pm$ SEM of three independent experiments. Differences were determined by a two-way ANOVA followed by Dunnett's *post-hoc* test. $^{*}p<0.05$ vs. control.

COL2A1 and aggrecan in a human chondrosarcoma cell line, SW1353 [34]. However, Zhou *et al.* showed that 24 h treatment with 100 μg/ml AGEs reduced the viability of SW1353 cells [35]. In the present study, 48 h treatment with 100 μg/ml AGE3 significantly reduced the viability of the human chondrocyte-like cell line OUMS-27 (Fig. 5). Among individuals with advanced diabetes, the serum AGE levels were 82.8 ± 9.4 μg/ml[36] with intra-articular levels potentially being significantly lower. These results emphasize that while high concentrations of AGEs may compromise chondrocyte viability, the concentrations used in our present investigation better reflect physiological conditions, allowing us to explore the catabolic effects without inducing cell death.

RAGE is the most extensively studied receptor in AGE-related investigations [37]. RAGE knockout mice are considerably protected against the development of OA [38]. TLR4, FEEL1, and LOX1 receptors have also been identified as AGE-binding receptors [39]. Our present findings revealed that the expression level of *RAGE* was upregulated after 2 weeks of stimulation with 10 μg/ml AGE3, whereas the expression levels of other receptors, including *TLR4*, *FEEL1*, and *LOX1*, remained unaffected (Fig. 3A). This indicates that RAGE is the primary receptor mediating the effects of AGE3 in OUMS-27 cells, making RAGE a key target for potential therapeutic interventions in patients with OA or RA.

To further substantiate our findings, we used FPS-ZM1, a high-affinity antagonist of RAGE. Pretreatment with FPS-ZM1 almost completely abolished the AGE3-induced degradations of COL2A1 and aggrecan (Fig. 3B-D). Similarly, other RAGE inhibitors such as salicin [40] and saxagliptin [41] have been successfully used to prevent cartilage degradation in a mouse model of OA and in patients with OA. AGEs in the ECM were reported to promote inflammation and cartilage degradation via RAGE. Evidence from both *in vivo* and *in vitro* studies indicates that AGEs are among the major players responsible for disrupting cartilage hemostasis by inducing the activity of inflammatory mediators, including proteases [42, 43]. These results highlight the importance of RAGE as a mediator of cartilage degradation and suggest that administration of RAGE inhibitors may be a promising therapeutic strategy for preventing AGE-induced cartilage damage in patients with OA or RA.

Notably, 24- or 48-h stimulation with 100 μg/ml AGE3 has been reported to increase the expression levels of MMP1, MMP3, MMP13, ADAMTS4, and ADAMTS5 by up to fourfold [43]. In contrast, in our experimental setup, stimulation with 10 μg/ml AGE3 for 1-24 h or 1-8 weeks did not alter the protease expression (Fig. 4A, B). Although no change was observed even at 100 μg/ml AGE3, AGE3 stimulation at doses > 500 μg/ml resulted in an increase in *MMP* expression (data not shown). Further studies should be conducted to clarify the effects of AGEs on MMP expressions. Together the past findings and our present results suggest that although low concentrations of AGE3 do not significantly impact the expressions of MMPs, higher doses may trigger protease activity, necessitating further investigation into the dose-dependent effects of AGEs on cartilage degradation.

Our study demonstrates that 4 weeks of 10 μg/ml AGE3 stimulation has deleterious effects on cartilage, highlighting the critical role of both the duration and the concentration of AGE exposure. These insights are crucial for understanding how long-term, even low-dose AGE exposure may contribute to cartilage degeneration and the pathogenesis of rheumatic diseases such as OA and RA.

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