Advanced glycation end-products downregulating intervertebral disc cell production of proteoglycans in vitro

KIMIYA YOKOSUKA, M.D., JIN SOO PARK, M.D., PH.D., KOTARO JIMBO, M.D., PH.D., KEI YAMADA, M.D., PH.D., KIMIYA SATO, M.D., PH.D., MIKIO TSURO, PH.D., MASAYOSHI TAKEUCHI, PH.D., SHO-IICHII YAMAGISHI, M.D., PH.D., AND KENSEI NAGATA, M.D., PH.D.

Departments of Orthopedic Surgery and Internal Medicine III, Kurume University School of Medicine, Kurume; and Department of Pathophysiological Science, Faculty of Pharmaceutical Science, Hokuriku University, Kanazawa, Japan

Object. The authors sought to clarify the role, if any, of advanced glycation end-products (AGEs) in disc degeneration.

Methods. Intervertebral discs were analyzed for the presence of AGEs and of their receptor (RAGE) by immunohistochemical analysis. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed to detect any RAGE gene expression, and real-time PCR was used to quantify messenger RNA (mRNA) levels of aggrecan and collagen types I and II in nucleus pulposus cells treated with AGEs. Aggrecan protein concentration was determined by enzyme-linked immunosorbent assay.

Immunohistochemical analysis revealed that AGEs and RAGE were localized in the nucleus pulposus of the intervertebral disc. Advanced glycation end-products were found to significantly suppress the expression of aggrecan at both mRNA and protein levels in a dose- and time-dependent manner. The levels of collagen types I and II remained unchanged after treatments with AGEs.

Conclusions. These results suggest that the accumulation of AGEs and their interaction with their receptor in the nucleus pulposus might result in the downregulation of aggrecan production responsible for disc degeneration.

KEY WORDS • advanced glycation end-product • advanced glycation end-product receptor • aggrecan • intervertebral disc degeneration • cadaver

Disc degeneration is a general term used to describe the complex involutional process that occurs in the intervertebral disc, which starts with cellular and biochemical changes and can eventually progress to a diseased state. Factors associated with disc degeneration include age, heredity, mechanical factors (such as abnormal loading due to torsion or compression), cigarette smoking, excess weight, and diabetes.\(^2,10,12,16,20,23\) The biological and pathological processes that occur during disc degeneration, however, remain poorly understood.

An important factor in disc degeneration is a decrease in the rate of production of aggrecan, which is a major proteoglycan of the intervertebral disc ECM.\(^1,5\) The loss of aggrecan occurs over a long period and is thought to result from a relatively minor imbalance between breakdown and production.\(^21,22\) The cause of the reduction in aggrecan synthesis remains unclear.

Advanced glycation end-products, which result from nonenzymatic glycation or the spontaneous reaction of reducing sugars with proteins, accumulate in all tissues with age. The buildup of AGEs is most pronounced in chondrocytes that contain long-living proteins, such as collagen and proteoglycan.\(^1,6,30\) It has already been reported that a high AGE accumulation leads to downregulation of proteoglycan production and the development of osteoarthritis in articular cartilage.\(^8,9,15,19,24,32\)

We hypothesized that the accumulation of AGEs in the nucleus pulposus might result in the downregulation of aggrecan production responsible for disc degeneration. As a first step to investigate this hypothesis, we evaluated how AGE accumulation influenced the biological function of the nucleus pulposus in vitro.

Materials and Methods

Cell Harvesting and Culturing

Lumbar intervertebral disc specimens were obtained at the postmortem examination within 6 hours of death. Informed consent of family members and the approval of the Kurume University Ethical Committee had been obtained. The donors were five men, ranging in age from 60 to 79 years, none of whom had had a clinical history of diabetes mellitus or had undergone any lumbar operation. Full-
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thickness intervertebral disc samples were taken from the L4–5 segment and were white or yellow in color. Because it is difficult to surgically acquire enough nucleus pulposus tissue in a lump sample without including anulus fibrosus tissue and because the samples were obtained after death, we were unable to use magnetic resonance imaging to evaluate possible disc degeneration.

The nucleus pulposus tissue was finely ground, while viewed though a stereo-microscope on a clean bench. It was then isolated in 2 mg/ml pronase for 90 minutes and in 500 μg/ml collagenase for 180 to 360 minutes, and the cells were subsequently isolated. Cell culture was performed in Dulbecco modified Eagle medium/F-12 medium containing 10% fetal bovine serum, 50 μg/ml ascorbic acid, and antibiotics (100 U/ml streptomycin and 100 U/ml penicillin). The medium was changed every 2 days, and two replicate samples were seeded onto plastic six-well plates (24 × 10^6 cells/2 ml medium in each). The samples were confirmed as containing chondrocyte-like cells by microscopic examination.

Histological Preparation

Sections were obtained from each disc and embedded in paraffin. The entire disc, including the endplates, was fixed in 10% formalin neutral buffer solution, decalcified with 10% ethylenediaminetetraacetic acid (pH 6.5), serially dehydrated in ethanol, and embedded in paraffin. The tissue blocks were then sectioned transversely at a thickness of 5 μm, mounted, and stained pseudoperoxidase with 3',3-diaminobenzidine HCl anti–mouse (Code No. KH001; Transgenic Corp., Kumamoto, Japan) and RAGE (1:500 dilution; Lot No. HKH02; Technne Corp., Minneapolis, MN). Negative controls involved the replacement of the monoclonal primary antibodies with mouse immunoglobulin G (Dako, Carpinteria, CA) at equal protein concentrations. Previously assessed samples were included in each staining to ensure consistency between the runs. After washing, the sections that were positive for AGEs and RAGE were incubated in labeled polymer horse-radish peroxidase (3’3-diaminobenzidine HCl) anti–mouse (Code No. K4000; Dako) using the Polymer-Immuno Complex (liquid 3’3-diaminobenzidine HCl substrate chromogen system; Code No. K3466; Dako) for 30 minutes at room temperature. The sections were then counterstained with hematoxylin, dehydrated, and mounted in Diatax (AB WILH, Becker, Sweden).

Preparation of AGEs

We have previously shown that glyceraldehyde-derived AGEs exert various biological activities through their interaction with specific RAGES.27–29 Glyceraldehyde-derived AGEs were prepared as described previously.

Messenger RNA Expression

Cells were stimulated in serum-free medium with 0.1, 1, or 10 μg/ml AGE, or nonglycated bovine serum albumin (control) for 6 days at 37°C in plastic six-well plates. The medium was changed every 2 days. Cells were collected and pooled from two wells to form one sample before the total RNA was purified using an RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. The RNA concentration was determined by ultraviolet extinction at 260 nm. The RNA sample was reverse transcribed to complementary DNA by using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) with TaqMan Reverse Transcription Reagents (Applied Biosystems). The reverse-transcriptase conditions were as follows: 25°C for 10 minutes, 45°C for 30 minutes, and 95°C for 5 minutes.

Reverse Transcriptase PCR

The RNA from cells that had not been stimulated by AGEs was reverse transcribed and then amplified with PCR by using the RAGE primer pair (Table 1). The PCR conditions were as follows: 35 cycles at 95°C for 1 minute, 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute. The product complementary DNA bands were visualized by electrophoresis in a 3.0% agarose gel.

Quantitative Assay for mRNAs of Aggrecan and Collagen Types I and II

Real-time PCR was performed to quantify aggrecan and collagen types I and II mRNAs using primers synthesized according to the published human sequences (Table 1). The 5700 Sequence Detection System (Applied Biosystems) was used with the SYBR Green PCR Master Mix (Applied Biosystems). The conditions for real-time PCR were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds, and 62°C for 1 minute without extension. The final two stages of this process were repeated for 40 cycles. The volume of the aggrecan mRNA was measured and normalized by β-actin with an internal standard, according to the delta-delta computed tomography method.26 The findings were compared with those for nonstimulated cells.

Concentration of Aggrecan Protein

The aggrecan protein concentration in the cell medium was measured by the ELISA with the PG EASIA kit (BioSource Europe SA, Nivelles, Belgium), which is specific for human aggrecan, and a Model 550 Microplate Reader (Bio-Rad Laboratories, Hercules, CA).

Statistical Analysis

The data were compared using the Student t-test. The results were recognized as statistically significant at a probability level of less than 0.05.

Results

Existence of AGEs and RAGE

Reverse transcriptase–PCR analysis obtained a 325-bp product (Fig. 1), showing a clear expression of RAGE in human nucleus pulposus cells.

Nucleus pulposus tissue obtained in a 65-year-old male cadaver showed diffuse staining for RAGE, with strongly labeled focal areas around the circumference of the cell surface and nuclear membrane (Fig. 2A and B). In addition, this tissue showed staining for AGES with strongly labeled focal areas in the ECM and the margins of the tissue cleft (Fig. 2D). No staining was apparent in the negative control (Fig. 2C and E). Similar results were seen in the nucleus pulposus tissues obtained in all five cadavers.

Expression Levels of Aggrecan and Collagen Types I and II mRNA

Aggrecan mRNA expression levels in cultured nucleus pulposus cells were determined after 6 days of treatment with different doses of AGEs (Fig. 3A). In the presence of 0.1, 1, and 10 μg/ml AGEs, the aggrecan mRNA expression levels on Day 6 were suppressed in a significant, concentration-dependent manner to 82.4 (p < 0.05), 74.4 (p < 0.01), and 49.2% (p < 0.01), respectively, of the untreated control levels. The expression levels of collagen types I and II, by contrast, did not differ significantly from
the control levels after 6 days of treatment with AGE (Fig. 3B and C). In the presence of 0.1, 1, and 10 μg/ml AGEs, the collagen type I mRNA expression levels, relative to control levels, were 105.7, 101.5, and 90.1%, respectively, and the collagen type II mRNA levels were 95.5, 107.9, and 112%, respectively.

A time-course experiment was then conducted to determine the effect of the length of AGE stimulation on aggrecan expression (Fig. 4A). Advanced glycation end-products at 10 μg/ml were shown to have a significant cumulative effect on the aggrecan mRNA expression: the levels were suppressed in a time-dependent manner to 81.4 (p < 0.05), 66.7 (p < 0.01), and 42.2% (p < 0.01) of the untreated control levels, on Days 2, 4, and 6, respectively.

Concentration of Aggrecan Protein

After establishing that AGE stimulation downregulated the aggrecan mRNA expression in the nucleus pulposus cells, we next investigated whether this activity was correlated with a decrease in the secretion of aggrecan protein in the culture medium. The ELISA was used to quantify the level of aggrecan protein in the conditioned medium of nucleus pulposus cells cultured with different doses of AGEs for 6 days without a medium change. The aggrecan protein concentration showed a tendency to decrease in an AGE dose-dependent manner; a statistically significant reduction was induced by 10 μg/ml AGEs (p < 0.05; Fig. 4B). Aggrecan concentrations 1.01-, 0.95-, and 0.91-fold that of the untreated control were measured after incubation of nucleus pulposus cells with 0.1, 1, and 10 μg/ml AGEs, respectively.

In the light of these findings, we further examined the effect of the 10 μg/ml AGE stimulation period on aggrecan protein levels. Nucleus pulposus cells were cultured with 10 μg/ml AGEs for 2, 4, and 6 days with no medium change, and the resulting aggrecan protein concentrations were determined. The respective concentrations of aggrecan protein on Days 2, 4, and 6 were 0.97-, 0.86- (p < 0.01), and 0.75-fold (p < 0.05) that of the untreated control at the same time points (Fig. 4C).

The experiments on the aggrecan protein levels were undertaken with no change of medium. Although this raised the possibility that cell metabolic activity could decrease the pH of the medium with time and that the observed decrease in aggrecan protein could be due to the increased acidity of the medium over time, each day’s sample showed a significant decrease in the concentration of aggrecan in the medium compared with the control under the same conditions. These results indicated that the concentration of aggrecan protein tended to decrease in a manner that was dependent on the AGE dose and on the period of exposure to AGEs.

Discussion

Advanced Glycation End-Products

Advanced glycation end-products are protein modifiers that are formed when an amino acid and a carbonyl group of a reducing sugar react with one another (the Maillard reaction). They are known to accumulate in various locations in the body such as the skin, myocardial cells, and renal cells, and particularly in chondrocytes and interver-

![Fig. 1. Expression of the RAGE gene in nucleus pulposus cells as determined by RT-PCR, showing a 325-bp product.](image-url)
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Fig. 2. Photomicrographs of nucleus pulposus tissue obtained in a 65-year-old male cadaver. A and B: Immunohistochemical staining for RAGE (arrow); strongly labeled focal areas are visible around the circumference of the cell surface and nuclear membrane. C: Negative control. D: Immunohistochemical staining for AGEs; strongly labeled focal areas are visible in the ECM and the margin of the tissue cleft. E: Negative control.

tebral discs, where metabolic turnover is slow.\textsuperscript{4,18,25,33}

The effects of AGEs were initially studied with reference to diabetes mellitus, but attention has now turned to their relationship with the causes of other disorders, in an attempt to elucidate their biological activities. The accumulation of AGEs is one of the major age-related changes that occur in articular cartilage. These protein modifiers reportedly play a role in the onset and progression of osteoarthritis by inhibiting the production of proteoglycan and collagen in articular cartilage cells.\textsuperscript{7,8} Nucleus pulposus cells of the intervertebral disc are chondrocyte-like cells, and there is some resemblance in biological character between these cells and chondrocyte cells of articular cartilage. Despite progress in understanding the effects of AGEs on articular cartilage, relatively little attention has been focused on the effects of AGEs on the intervertebral disc.

Accumulation of AGEs in the Intervertebral Disc

In 1991, Hormel and Eyre\textsuperscript{11} reported that the brown degeneration observed in the nucleus pulposus area of the degenerated intervertebral disc was the result of an accumulation in protein glycation products (for example, AGEs), with specific fluorescence, which increased over time. Schleicher, et al.,\textsuperscript{25} have reported that the immunolocalization of AGE in normal fetal, juvenile, and adult tissues clearly revealed an age-dependent staining pattern; in addition, the normal nucleus pulposus cells were negative, whereas a slight to moderate positive staining was seen for degenerated intervertebral disc tissue. However, it was unclear from their study whether AGEs caused the disc degeneration or simply accumulated as a result of it. The authors of additional studies have since reported that AGEs accumulate in various tissues over time, although accumulation alone does not result in biological activity; rather, various biological activities occur only after AGEs react with RAGE.\textsuperscript{13,17,26}

In the present study, we have provided the first evidence that RAGE is present in the nucleus pulposus of the intervertebral disc. Because not only AGE but also RAGE exists in the nucleus pulposus, it is possible that the biological activities that occur in articular cartilage also take

Fig. 3. Bar graphs showing the effect of AGEs on aggrecan (A), collagen type I (B), and collagen type II (C) mRNA expression as determined by real-time PCR. Aggrecan mRNA decreased in a dose-dependent manner, whereas collagen types I and II were unaffected by AGE stimulation. The data are normalized to the corresponding values of the control group (mean ± standard deviation [SD]; five specimens for each). *p < 0.05 compared with the control group; **p < 0.01 compared with the control group.
place in the intervertebral disc. We have actually confirmed that AGEs significantly suppress the expression of aggrecan at both the mRNA and protein levels in a dose- and time-dependent manner. These results suggest that AGEs do not simply accumulate, but that accumulation of AGEs during the aging process might be a causative factor for the onset and progression of disc degeneration through inhibition of the production of aggrecan.

In the clinical setting, the extent of disc degeneration varies considerably among individuals, regardless of age or sex. It might be speculated that such variations are influenced by differences in the rate of AGE accumulation, but further investigation will be needed to clarify this point.

**Conclusions**

Our findings show that AGEs and RAGE were present in the nucleus pulposus of intervertebral discs. We have also demonstrated that AGEs suppress aggrecan expression at both the mRNA and protein levels. We propose the hypothesis that AGEs can accumulate over time in the nucleus pulposus and subsequently bind to the specific AGE receptor, thereby inhibiting the secretion of aggrecan. This might play an important role in the onset and progression of disc degeneration, similar to that seen in articular cartilage.

**References**


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Address reprint requests to: Jin Soo Park, M.D., Ph.D., Department of Orthopedic Surgery, Kurume University School of Medicine, 67 Asahimachi, Kurume City, Fukuoka 830-0011, Japan. email: jisopark@hotmail.com.