Effect of AGEs on Human Disc Herniation: Intervertebral Disc Hernia Is Also Effected by AGEs

MICHIYO TSURU, KENSEI NAGATA, ATSUO JIMI*, KOUJI IRIE**, AKIRA YAMADA*, RYOJI NAGAI#, SEIKOH HORIUCHI# AND MICHIO SATA$	extdagger$

Departments of Orthopaedic Surgery, Pathology*, Immunology*, Medicine#, Kurume University School of Medicine, Kurume 830-0011, **Department of Pathology, Kouseikan Hospital, Saga 840-8571 and #Department of Biochemistry, Kumamoto University Medical School, Kumamoto 860-0811, Japan

Summary: Currently, extracellular matrix MMP has been discussed in relation to the extrusion and spontaneous regression of the herniated mass observed in lumbar disc herniation. However, the question remains as to whether degenerated protein is really the cause of this condition's pathogenesis. We confirmed immunologically by means of electron microscopy that extrusion is caused by the AGEs (advanced glycation end products)-induced cross-linking of collagen, and that spontaneous regression is due to AGE receptors on macrophages. Further, AGEs were found to be already exposed during histogenesis, suggesting a relation to apoptosis. In lumbar disc herniation and aging, glucose-derived AGEs cross-link proteins and cause vascular tissue damage.

Key words AGEs, RAGE, human disc herniation, aging, glucose, IRS-1

INTRODUCTION

Advanced glycosylation end products (AGEs) form spontaneously from glucose-derived Amadori products and accumulate on long-lived tissue proteins. We succeeded in achieving in vitro establishment of human herniated intervertebral disc-derived chondrocytes and prepared an in vitro extrusion hernia model (KTN-1). Using this model, we immunologically investigated tissue degeneration using an electron microscope. In this study we evaluated AGE in terms of the following features: 1) The acidic condition of the isolated cells; 2) The survival of cells in a serum-free condition, considering the poor vascularization in the intervertebral discs; 3) In routine clinical practice, some patients with intervertebral disc herniation showing or having previously shown a high blood glucose level similar to the state of cataract patients. Intervertebral disc hernia patients have been found to have high concentrations of AGEs in tissue. AGE have been shown to account for numerous biological responses [1]. In addition to forming protein-to-protein crosslinks which underlie connective tissue aging, AGEs are also recognised by AGE-specific receptors and promote cytokine [2] and growth factor [3,4] release, as well as matrix protein synthesis [5]. Tissue-derived degradation products of AGE-modified proteins may be generated either by a specific macrophage AGE-receptor pathway or by extracellular proteolytic systems. Cells of the monocyte/macrophage lineage were the first found to display a surface receptor system mediating the binding and internalization of AGE-modified macromolecules. These receptors are structurally and functionally distinct from other known macrophage scavenger receptors [6,7]. This study is significant for hernia therapy in the near future in the context of an approach from sugar (cause), not aging (result).

MATERIALS AND METHODS

Tissues

Herniated intervertebral discs were obtained dur-
Ining surgery. Human fetal tissue was obtained by therapeutic abortion.

Immunohistochemistry

The paraffin sections were soaked in xylene to remove paraffin and rehydrated in a graded series of alcohol (100% to 70%). After deparaffinization, the sections were rinsed in tap water for 10 min. After trypsin treatment, endogenous peroxidase activity was quenched by immersion in 10 mmol/L phosphate-buffered saline (PBS; pH 7.4) with 0.3% hydrogen peroxide for 30 min. After being rinsed in 10 mmol/L PBS for 5 min, the sections were incubated for 30 min in diluted normal blocking serum, which was prepared from the species in which the secondary antibody was made. Polyclonal antibodies against AGE-receptor (RAGE) and monoclonal antibodies against AGE (Kumamoto Immunochemical Laboratory Co., Ltd.) were used as primary antibodies. The sections were incubated for 16 hs with a 1:100 dilution of primary antibodies at 4 °C. After rinsing in PBS, the sections were sequentially incubated in diluted biotinylated secondary antibody solution for 30 min. The reaction was visualized by immersing sections in diaminobenzidine hydrochloride with 0.6% hydrogen peroxide as substrate for 1-2 min. The sections were finally counterstained with Mayer’s hematoxylin to visualize the nuclei, then rinsed in tap water for 10 min, dehydrated in a graded series of alcohol (70% to absolute methanol) and through xylene, mounted. Paraffin-wax tissue sections were prepared at 5 μm on microscope slides coated with aminopropylethoxysilane (APES) and dried overnight at 37 °C for subsequent TUNEL staining and immunohistochemistry. TdT-mediated dUTP-FITC nick end-labelling (TUNEL) was performed using a commercial kit (Boehringer Mannheim Ltd.) and according to the manufacturer’s instructions. The cell suspension of heparanized peripheral blood was loaded carefully on three volumes of Ficoll sodium metrizoate. Cells were examined at three days post plating, at which time they had differentiated into monocyte derived macrophages, regardless of their incubation temperature.

Immunofluorescence staining

Frozen sections (8 μm) mounted on polylysine-coated slides (Fisher Scientific Co.) were fixed in 4% paraformaldehyde for 10 min at room temperature, and incubated with hyaluronidase (1 mg/ml) for 30 min at 37 °C. The sections were blocked in PBS containing 10% normal donkey serum for 1 h at 37 °C. All primary antibodies were diluted in PBS containing normal donkey serum (1%). RAGE was used at a dilution of 1:1000 and incubated with sections overnight at 4 °C. After washing in PBS, the sections were incubated sequentially with the appropriate secondary antibodies for 30 min at room temperature. After washing, the sections were mounted in fluorescent mounting medium and viewed on a Nikon Optiphot using DM510 filter cubes.

Electron microscopic immunohistochemistry

Samples were sliced, rinsed with PBS, and fixed with 4.0% paraformaldehyde/0.1% glutaraldehyde in PBS for 3 hs at RT with mixing. The fixed tissues were then rinsed in PBS and cut into smaller blocks [8]. Following microwave oven (Yokogawa Electric Corporation, Tokyo, Japan) irradiation, the sample bottles were kept on ice for 10 min [9]. After rinsing with a cold solution consisting of 100 mM lysine/100 mM sodium phosphate, pH 7.4/150 mM NaCl, the specimens were dehydrated in a graded series of cold ethanol, and embedded in LR-White at 50 °C. Ultrathin sections were cut on a MT-7000 ultramicrotome (Research and Manufacturing Company, Inc.) and collected on nickel grids with polyvinylformal membranes. The sections were immunostained with monospecific antibodies and a protein A-gold solution with 15 nm to 5 nm gold particles (EY Laboratories; San Mateo, CA) [10]. After staining with uranylacetate and lead citrate, the specimens were observed via a JEM 2000 electron microscope at an accelerating voltage of 100 kV. For preparation of the cultured cell specimen, the cell layers were removed from the dish and fixed in situ for 1 h at 4 °C with 4.0% paraformaldehyde/0.1% glutaraldehyde in PBS for 3 hs at RT. After washing overnight at 4 °C with 7.5% sucrose in 0.1 M sodium cacodylate at pH 7.4, the specimens were dehydrated in graded ethanol. Immediately before propylene oxide treatment, the samples were embedded in LR-White at 50 °C.

RT-PCR

Total cellular RNAs were extracted using Isogen (Nippon Gene Co., Tokyo, Japan) and cDNAs were synthesized from 5 μg of total RNA using RAV-2 reverse transcriptase (Takara, Japan) in the presence of random primers (Takara) in a 20 μl reaction volume at 42 °C for 60 min. One microlitre of cDNA solution was amplified by Taq polymerase (Takara) in a volume of 10 μl. For mRNA detection of IRS-1 and RAGE genes, the PCR procedure was performed
Fig. 1. A: Immunolocalization of AGE-producing cells in the degenerated or herniated intervertebral disc.
B: At higher magnification, the chondrocyte can be seen.

Fig. 2. H & E stain of formalin-fixed, paraffin-embedded sections of the liver (A). Immunolocalization of AGE-producing cells in the liver (B), the muscle (C), and the skin (D).
Fig. 3. Human fetal tissue obtained by therapeutic abortion. H & E stain of formalin-fixed, paraffin-embedded sections of the vertebral column (A, B). Immunolocalization of AGE-producing cells in the vertebral column (C).

Fig. 4. Human fetal tissue obtained by therapeutic abortion. Immunolocalization of RAGE-producing cells in the vertebral column (A, B).
with 36 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 90 s, with a predenaturing time of 2 min and a final extension time of 5 min. The primer sequences were IRS-1, 5'-CTCGTCAAAGCTATGTGGATTCC-3' (sense) and 5'-GTTGCTTCTGGAAGTTGATGC-3' (antisense); RAGE, 5'-GCAGTAGTAGTTGCTCAAAAC-3' (sense) and 5'-GTGTCAGGTTGTTTAATCATCA-3' (antisense).

RESULTS

We used specific anti-AGE antibodies in an immunohistochemical study of intervertebral disc specimens obtained from herniation individuals. Figures 1A and B from a 67-year-old herniated patient show a representative section that was stained for tissue AGEs. Positive staining was observed with control tissue that had been preabsorbed with AGES (Fig. 2), indicating that the anti-AGE staining was specific. AGES in positively stained sections were localized primarily within the liver (Fig. 2B), the smooth muscle cells (Fig. 2C) and the skin (Fig. 2D). As a control, human fetal (aborted) tissue obtained from 8W of the vertebral column was immunohisto logically stained, as shown in Fig. 3. Of three adjacent sections, the first was stained with hematoxylin and eosin (H & E: Figs 3A and B), the second was used for immunohistochemistry (AGE: Fig. 3C), (RAGEs: Figs 4A and B). The second sections were
boiled in 0.1% sodium citrate for 5 min and incubated with goat IgG anti-RAGE monoclonal antibody at 1:100 dilution for 6 hs. After incubation, the sections were washed with PBS, incubated with fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG antibody (1:1000) and observed with a darkfield fluorescence microscope (Figs 5A and B). The same tissue was also stained with apotac, and as shown in Fig. 6, positive staining was obtained. This finding indicated that AGEs are related to apoptosis. Figure 7 shows AGE receptors expressed by macrophages. In herniated intervertebral disc patients complicated by diabetes, the expression of the RAGE gene was 1328bp (arrow) (Fig. 8a), and the IRS-1 gene was 243bp (arrow) (Fig. 8b).

DISCUSSION

In humans cell proliferation and death are ingeniously controlled to maintain homeostasis. Many cells are removed by cell death during embryogenesis. In a mature body, cells constituting organs maintain functions by constantly balancing proliferation and death. In organs, aged cells are removed by cell death and replaced by newly growing cells. This cell death has been previously scheduled, and is called programmed cell death. Programmed cell death is induced by physical and chemical factors. In this study, a relationship between programmed cell death and AGEs was suggested. During the early step of glycosylation, the reaction progresses in a manner dependent on saccharide concentration and reaction time. In the subsequent late step, an irreversible reaction occurs. In patients in whom the blood glucose level had been high in the past, the incidence remained high even though the blood glucose level is currently controlled, suggesting that AGEs affect a gene and that the effect is memorized. Our study may be significant in elucidating another pathological state of lumbar disc hernia. In summary, we have identified an inducible AGE as a novel mechanism for the cooperative interaction between tissue macrophages and lymphocytes during tissue homeostasis or repair. Under conditions of excessive AGE protein/lipid accumulation (in aging or chronic diabetes), this orderly system may be disturbed so that inappropriate lymphokine activity, in synergy with macrophage-derived cytokine activity, could lead to tissue injury. AGEs and insulin induce a wide variety of growth and metabolic responses and play important roles in the anabolic regulation of bone metabolism.

ACKNOWLEDGMENTS: The authors would like to thank Prof. Zenji Makita (Division of Endocrinology and Metabolism, Department of Medicine, Kurume University School of Medicine) for suggesting this problem and for stimulating interest in it. They are grateful to Mr. K Yoshida, Mrs. M Nagao, Mrs. Y Kawahara, Miss. R Matsuda, Miss. C Hijikata, Miss. C Hashimoto and R Yamagata (Department of Orthopaedic Surgery, Kurume University School of Medicine) for helpful suggestions and observations and a critical reading of the manuscript.
REFERENCES


