Immunohistochemistry of chondromodulin-I in the human intervertebral discs with special reference to the degenerative changes

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Summary

The expression of the matrix protein chondromodulin-I has been studied in human intervertebral discs of 101 people using immunohistochemical analyses. The purpose of this report is to present data on the metabolic changes that were found to occur in the chondrocytes of intervertebral discs during development and aging. Chondromodulin-I was highly expressed during the gestational period and gradually decreased after maturation. It was detected in both the extracellular matrix and chondrocytes in the zone of hypertrophic cartilage, the zone of proliferative cartilage and the zone of resting cartilage in fetal discs. It was also present in the annulus fibrosus, nucleus pulposus and end-plate cartilage in mature discs. In degenerative discs, chondromodulin-I immunoreactivity tended to be elevated in the remaining chondrocytes. Our findings suggest that the expression of the protein is developmentally regulated and upregulated through a defense mechanism against the degenerative processes of the aged intervertebral disc.

Introduction

The mobility and stability of the human spine depends to a large extent on the integrity of the intervertebral discs. Although it is known that in intervertebral disc disease, a common pathway of aging and degeneration of the disc results from mechanical stress on either the nucleus pulposus or the annulus fibrosus, or both. However, the underlying mechanism has not yet been clearly defined (Dupuis et al. 1985, Frymoyer et al. 1979, Lindahl 1996). It is clinically important to study the mechanism of disc degeneration. Degenerative changes of the disc are considered to be related to the changes in the cartilage plate (Coventry et al. 1945, Nachemson et al. 1970). Moreover, a loss of functional end-plate cartilage has been associated with gross morphological changes in the disc and the adjacent vertebral body (Maroudas 1988). The cause of disc degeneration is due to the end-plate cartilage becoming irregular and often broken off by the replacement of fibrous connective tissue including blood vessels.

Cartilage is unique among the tissues of mesenchymal origin in that it is resistant to vascular invasion due to an intrinsic angiogenic inhibitor. Hiraki and others found the inhibitor to be identical with chondromodulin-I (ChM-I) (Hiraki et al. 1996). ChM-I was isolated from fetal bovine cartilage, and binds to heparin with high affinity (Hiraki et al. 1991). It is a 25 kDa glycoprotein generated from a larger transmembrane precursor after post-translational modification and proteolytic cleavage at the processing signal site (Hiraki et al. 1997b). The carboxyterminal portion of the molecule is secreted into the extracellular matrix. ChM-I expression has been described mainly in cartilage (Hiraki et al. 1991, 1999, Shukunami et al. 1999). The protein stimulates the growth of rabbit growth plate chondrocytes in the presence of fibroblast growth factor-2 (FGF-2), as well as proteoglycan synthesis by the cells (Hiraki et al. 1996). It stimulates the anchorage-independent growth of chondrocytes in agarose (Inoue et al. 1997), whereas it inhibits the growth and tube morphogenesis of cultured vascular endothelial cells (Hiraki et al. 1997a). ChM-I is a bifunctional growth regulator depending on the target cell type.

The factors related to disc degeneration and angiogenesis have yet to be clarified. We need, therefore, to elucidate the relationship between ChM-I and angiogenesis. This paper reports an analysis of disc degeneration with age using ChM-I immunohistochemistry.

Materials and methods

Tissue samples

The lumbar spines of 101 autopsied subjects in the files of the Departments of Pathology and Neuropathology, Kyushu University were examined. The ages of the patients at the time of death ranged from 18 gestational weeks to 96 years.
Cadaveric lumbar spines were excised from 15 fetuses (age of gestation, 18–37 weeks), 5 infants (0–5 months), 13 young adults (20–49 years), and 10 adults (50–59 years) to obtain normal intervertebral discs. 58 lumbar spines were removed from older individuals aged 60–96 years to find degenerative intervertebral discs. At the time of autopsy, the vertebral column including the anterior and posterior elements were removed en bloc and fixed with buffered 10% formalin. A sagittal midline slice was made from each specimen, and it was transected at the centre of each vertebral body. The gross features of the slices were noted and each disc was photographed in colour. The slices were embedded in paraffin wax and routinely stained with haematoxylin and eosin (HE), alcian blue and Masson’s trichrome stain.

Immunohistochemistry

Immunohistochemical staining was performed in all cases, using the labelled biotin–streptavidin method. An antiserum against human ChM-I was raised in a rabbit by injecting a synthetic oligopeptide corresponding to amino acids 8–33 of the mature human ChM-I protein sequence (PTTTKPHSLGPRSNPAGRLNNEPTR) (Hiraki et al. 1999).

Sections were cut at a thickness of 6 µm and then deparaffinized in xylene, dehydrated in ethanol, and incubated with 0.3% hydrogen peroxide in absolute methanol for 30 min at room temperature to inhibit endogenous peroxidase. To enhance the immunoreactivity for ChM-I, the sections were treated either with 0.05% trypsin (Mochida, Japan) in phosphate-buffered saline (PBS) for 30 min at 37 °C or with 3500 units/ml testicular hyaluronidase (type V, Sigma) in PBS for 50 min at 37 °C (Inkinen et al. 1996, Tammi et al. 1999). After washing with Tris–HCl buffer (50 mmol/L Tris–HCl, pH 7.6), the sections were then incubated with diluted primary antibodies at 4 °C overnight. The following steps were carried out with species-specific biotinylated secondary antibodies and horseradish peroxidase-labelled streptavidin according to the manufacturer’s instructions (Amersham, Buckinghamshire, UK). The coloured reaction product was developed with 3,3'-diaminobenzidine tetrahydrochloride. Finally, the sections were counter-stained lightly with haematoxylin.

Absorption of antibodies

The specificity of the ChM-I antibody was tested by preabsorption tests using recombinant human ChM-I (rhChM-I) protein. rhChM-I was expressed in Chinese hamster ovary cells and purified from the culture supernatant to homogeneity by sequential chromatography (Hayami et al. 1999, Hiraki et al. 1999). The antibodies were absorbed with rhChM-I. The antibodies were diluted 1 : 250 in 1 ml TBS-Tween (50 mmol/L Tris–HCl, pH 7.6, 0.5 mol/L NaCl, 0.05% NaN₃, and 0.05% Tween 20) containing 50 µg rhChM-I. After 1 h at room temperature, the serum was centrifuged at 100,000g for 1 h at 4 °C. The supernatant solution was then used for immunohistochemical staining (Iwaki et al. 1989).

Western blot analysis

A Western blot analysis was performed on the frozen tissue specimens from two cases to determine the specificity of ChM-I antibody. At autopsy, the disc tissue specimens were removed and frozen in liquid nitrogen and thereafter kept at −80 °C. The frozen samples were homogenized and solubilized in lysis buffer (2% sodium dodecyl sulphate, 2 mM EDTA, 2 mM phenylmethylsulphonyl fluoride, 50 mM Tris–HCl, pH 6.8). The protein concentrations were determined by a modified Lowry procedure using bovine serum albumin as the protein standard (Markwell et al. 1978). A sample buffer was added to this mixture. The samples were then boiled for 4 min. Electrophoresis of the protein samples (50 µg) was performed on 13% polyacrylamide gel containing 0.1% sodium dodecyl sulphate. The proteins were then electrophoretically transferred from the gel to a polyvinylidene difluoride membrane (Millipore, Nihon Millipore Kogyo K.K., Japan). The blotted membrane was washed and blocked with TBS-Tween containing 5% low fat milk. The membrane was then incubated overnight with ChM-I (1 : 1000). The blot was washed in TBS-Tween and incubated with alkaline phosphatase-conjugated secondary antibody (Promega, Madison, WI) diluted 1 : 7500 in TBS-Tween containing 5% low fat milk for 1 h. The colour reaction was developed in AP buffer (100 mmol/L Tris–HCl, pH 9.5, 100 mmol/L NaCl, 5 mmol/L MgCl₂) containing 0.66% nitroblue tetrazolium and 0.33% 5-bromo-4-chloro-iodoindol phosphate.

Data collection and analysis

Fifty-eight lumbar spines removed from older individuals aged 60–96 years were assessed by macroscopic inspection and divided into four groups according to Nachemson’s scheme for grading disc degeneration. Group 1: Discs without changes visible to the naked eye. In these cases a gelatinous shiny nucleus pulposus was seen; it was delimited from the annulus fibrosus, which was free from macroscopic ruptures. Group 2: Discs which showed macroscopic changes in the nucleus pulposus. The nucleus was somewhat more fibrous, but could be clearly distinguished from the annulus, which was intact. Group 3: Specimens which showed macroscopic changes in both the nucleus pulposus and the annulus fibrosus. The nucleus in these discs was more fibrotic but still soft. The boundary between the nucleus and annulus was no longer so distinct, but it could still be seen. Changes in the annulus fibrosus consisted of isolated fissures. Group 4: Specimens which showed more severe macroscopic changes. The discs in this group exhibited fissure formation and cavities in both the nucleus and the annulus. Marginal osteophytes were often found in adjoining vertebrae (Nachemson 1960). The relative amount of ChM-I deposition was graded using a semiquantitative scoring system. In the end-plate cartilage, nucleus pulposus and annulus fibrosus, we estimated the relative number of stained cells per total cells. (+) indicates less than 25% of the cells are positive; (+++), 25–50%; and (++++) more than 50% of the cells are positive.