Expression of lamin A/C protein in degenerated human intervertebral disc

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European Review for Medical and Pharmacological Sciences 2018; 22: 7607-7613

Abstract. – OBJECTIVE: This study aimed to evaluate the expression characteristics of lamin A/C proteins in intervertebral disc degeneration (IVD) specimens from patients with different degeneration grades. Lamin A/C proteins have been shown to result in age-related changes in the osteoarticular system. However, the expression characteristics of these nuclear proteins in degenerated human IVD tissues have not been explored previously.

PATIENTS AND METHODS: Degenerated human IVD tissues were obtained during spinal surgery. Articular cartilage samples after total knee replacement surgery were used as controls. Sections of these tissues were stained with hematoxylin and eosin, Masson, safranin O, and immunostained using lamin A/C antibody. Western blot was performed to evaluate lamin A/C expression in IVD tissues. Lamin A/C expression was analyzed based on different degeneration grades.

RESULTS: In patients with IVD degeneration, mild or moderate degenerative discs contained high amounts of lamin A/C proteins. Lamin A/C expression was primarily localized in the nuclear envelope of IVD cells, and associated with apoptosis in cell nuclei, as determined by immunostaining and TUNEL assay.

CONCLUSIONS: This paper is the first to report that lamin A/C proteins are present in IVD tissues and its expression may be related to disc degeneration.

Key Words: Lamin A/C, Osteoarticular system, Intervertebral disc degeneration (IVD), Spinal surgery.

Introduction

Lamin A/C is a member of the nucleoprotein family that belongs to the type V intermediate filament. Lamin A/C mainly distributes below the nuclear intima and plays an important role in maintaining nuclear shape and structure. The hydrolysis of lamina proteins in cell apoptosis has been elucidated. Since cell apoptosis plays an essential role in intervertebral disc degeneration (IVD), we were interested in elucidating the connection between lamin A/C hydrolysis and IVD.

Several studies have confirmed that lamin A/C is linked to bone and cartilage metabolism. In bone research, some scholars reported that bone intensity and quantity are significantly lower in lamin A/C knockout mice than in normal mice. In cartilage metabolism, damage to or interference with lamin A can lead to cartilage cell death. Further bone and cartilage studies showed that lamin A/C expression is significantly different in young and old mice, and its expression decreases with increasing age. Lamin A/C plays an important role in bone and joint systems. However, the expression of lamin A/C in degenerated human IVD, which is similar to cartilage, has not been studied in detail before. This study aimed to detect the expression characteristics of the lamin A/C in degenerated human IVDs.

Patients and Methods

Patient Selection and Sample Collection

This study was approved by the institutional Ethics Committee. Informed written consent was obtained from each participating patient. The general clinical data for the cohort are shown in Table I. Degenerative IVD were obtained from patients subjected to spinal fusion surgery. All patients underwent routine lumbar magnetic resonance imaging (MRI) examination. The IVDs
were classified according to the modified Pfirrmann evaluation. IVD samples classified in the degeneration Grades I to III were grouped together. Samples in Grades VI to VIII were placed into another group (Table I).

Three specialists assessed the IVD degeneration grades. The opinion of two doctors was sufficient to classify the samples; when the three doctors disagreed on the grade of degeneration, the specimens were excluded from this study. All the samples were collected in our department from June 2014 to October 2014. Samples were rinsed in phosphate-buffered saline (PBS) and sheared into pieces under a microscope. Samples were fixed in 4% paraformaldehyde or 2.5% glutaraldehyde for 3 h. Cartilage for the control group was selected from patients undergoing knee replacement surgery.

**Transmission Electron Microscopy (TEM)**

The IVD block (1 mm³ volume) was fixed for 3 h using 2.5% neutral glutaraldehyde and rinsed six times using PBS for 30 min each time. Subsequently, 1% osmic acid was used for fixation for 1.5-2 h. PBS was used to rinse the samples for three times. The above steps were carried out at 4°C. After fixation, the samples underwent routine gradient dehydration, permeabilization at room temperature for 3-4 h with resin mix (100% acetone: Epon 812 = 1:1), and encapsulation in the mold. After routine consolidation in the oven, a Leica UC7 ultramicrotome was used for sectioning (thickness 50-70 nm). Finally, uranium-lead double staining was performed (2% uranyl acetate staining for 30 min; lead citrate staining for 5 min). The sections were observed under a JEM 1400 TEM (Jeol, Japan).

**Histology**

IVD samples were fixed in 4% polyformaldehyde for 24 h, decalcified in 10% EDTA, and embedded in paraffin. Four serial sections (5 μm thick) per sample were cut with a microtome (Leica, Wetzlar, Germany). To observe cell morphology, density, and collagen expression, three sections from each specimen were stained with hematoxylin and eosin, safranin O-fast green and Masson.

**Immunostaining for LMNA**

For immunohistochemistry analysis, paraffin sections were de-paraffinized with xylene and serially rehydrated. After treatment with EDTA (pH 9.0) in a microwave oven for 15 min for antigen retrieval, endogenous peroxidase activity was blocked with 3% H₂O₂ for 10 min at room temperature. A 1 h blocking step was conducted with 3% goat serum and 1% bovine serum albumin (BSA) in PBS. Rabbit anti-human lamin A/C at 5 μg/mL (Abclonal Technology, Rocky Hill, CT, USA) was applied overnight at 4°C, following by washing with PBS. Goat anti-rabbit secondary antibody (1:5000 dilution) was applied for 1 h at

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<th>MPE</th>
<th>Grade I-III</th>
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<tr>
<td>1</td>
<td>Male</td>
<td>24</td>
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<td>35</td>
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<tr>
<td>3</td>
<td>Male</td>
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**Table I.** Detail of patients.
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room temperature. After washing, a chromogenic reaction was performed using a Histostain ABC kit (Boster, Wuhan, China) according to the manufacturer’s instructions.

**Cell Death Assay**

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) experiment-included routine de-waxing of paraffin sections, protease K antigen repair, and incubation at room temperature for 10 min, with 50-100 µL of membrane rupture liquid. According to the Roche TUNEL kit (St. Louis, MO, USA) appropriate amounts of reagent 1 (TdT) and reagent 2 (dUTP) were mixed at a ratio of 1:9 and added in the circle to cover the tissue. The sections were placed flat in a wet box and then incubated in a 37°C water bath for 60 min. PBS (pH 7.4) was added three times to wash cells on a shaking rotator. Subsequently, 3% hydrogen peroxide solution prepared with methanol was added, and samples were incubated in the dark at room temperature for 20 min to block endogenous peroxidase activity. Finally, an appropriate amount of reagent 3 (converter-POD) was added to all sections to cover the tissues. The sections were laid flat in the wet box and incubated for 30 min in a 37°C water bath pot. The sections were placed in PBS (pH 7.4) on the shaking rotator and washed three times. Routine diaminobenzidina (DAB) coloring and hematoxylin nuclear counterstaining were performed. The cell apoptosis of sections was observed under a microscope.

**Quantitative Western Blot Analysis**

IVD samples were placed on ice immediately after surgery and rinsed twice with PBS. To isolate nuclear protein, tissues were lysed using a nuclear and cytoplasmic protein extraction kit (Boster, Wuhan, China) according to the manufacturer’s instructions, followed by centrifugation at 12,000 rpm to extract the final supernatant. For Western blot, 25 µg of proteins were resolved on 10% SDS-PAGE and transferred by electroblotting to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked in 5% Bovine Serum Albumin (BSA) in Tris-buffered saline with 0.1% Tween-20 at room temperature for 2 h and then incubated with anti-lamin A/C (1:1000 dilution) and anti-Histone 3 (1:5000 dilution, ABclonal Technology, Rocky Hill, CT, USA) as an internal control overnight at 4°C. Membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG, followed by detection using an Odyssey Infrared Imaging System (LI-COR, Bioscience, San Jose, CA, USA). This experiment was repeated four times. Protein bands were analyzed using the LI-COR imaging software and exported to Microsoft Excel. Signal intensities among different degrees of degeneration were compared using the nonparametric test (Kruskal-Wallis test) and Dunnett’s test.

**Statistical Analysis**

SPSS 19.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. All quantitative data were expressed as mean ± standard deviation. Comparison between groups was done using One-way ANOVA test followed by Post-Hoc test (Least Significant Difference). p-values < 0.05 were considered statistically significant.

**Results**

**Lamin A/C Expression in IVD**

Figure 1 shows the immunohistochemical results of lamin A/C in degenerated human IVD. Lamin A/C accumulated around the nucleus of the nucleus pulposus tissue (Figure 1 A1-A2, solid black arrows). Lamin A/C expression was lower in the annulus fibrosis than in the nucleus pulposus (Figure 1 A1-A2, dashed black arrow). The immunohistochemical results showed that the expression of lamin A/C in mild degenerative IVD patients was significantly higher than in the control group (\(p < 0.05\)) (Figure 1 A1-A2). Cartilage from human osteoarthritis after knee arthroplasty was used as positive control group (Figure 1C). The negative control group (without primary antibody incubation) did not show non-specific staining (Figure 1C).

**Apoptosis in IVD Tissue**

TUNEL detection reflects the rupture of nuclear DNA during cell apoptosis. Staining degenerated IVD with TUNEL showed stronger signal in the severe degenerative group (Figure 1, dashed black arrow) compared to the mild degenerative IVD group (Figure 1B, solid black arrow). The negative nucleus is shown in Figure 1B (solid white arrow).

**Morphological Changes in Nuclear Envelope**

To further validate the structural changes due to apoptosis, we conducted TEM of the degen-
erated IVDs. The results of TEM showed the change in the cells of the nucleus pulposus due to apoptosis (Figure 2A) and necrosis (Figure 2B). Nuclear peripheral structures dissolved and then disappeared (black arrow).

Correlation of Lamin A/C Expression with IVD Degeneration

We next performed Western blot to assess in a more quantitative assay the amount of lamin A/C protein expressed in the degenerated IVDs. Lamin A/C expression decreased with the progression of IVD degeneration. The lamin A/C expression from the mild degenerated group (Grades I to III) was significantly higher than in the samples from the strong degenerated group (Grades VI to VIII) (p < 0.05) (Figure 3, left). Lamin A/C expression was slightly lower in the mild degenerative group compared with the control group, but the difference was not significant (p > 0.05). To further explore the correlation between the lamin A/C protein and IVD degeneration, Western blot was repeated four times for each sample. Figure 3 (right) displays the difference in protein expression intensity between the different degenerative groups.

Discussion

In this study, we analyzed the expression of the lamin A/C protein in degenerating IVD and found that its expression in mild cases was higher than that in the more serious patients. These results suggested a potential role for lamin A/C in spinal IVD degeneration. Earlier studies have shown...
showed that mutant lamin A causes changes in the skeletal system in patients with premature senility syndrome or progeria. Subsequent studies\(^8,11\) showed that the absence of the lamin A/C or changes in age alters bone and cartilage metabolism. Some studies\(^12\) suggest that the integrity of lamin A/C has a protective effect against apoptosis of osteoblasts. By contrast, Attur et al\(^5\) found that lamin A/C expression increases in osteoarthritic cartilage and the excessive accumulation of lamin A/C causes early chondrocyte apoptosis. In our study, cell number and lamin A/C expression were higher in mild degenerative IVD than in the control group. Moreover,
lamin A/C downregulation was accompanied by increased cell death. Further electron cytology observations showed that the morphology of karyotheca changed from cell apoptosis to death. Overall, it seems that both over- and under-expression of lamin A/C can cause changes in skeletal system metabolism. Thus, normal expression of lamin A/C plays an important role in maintaining cell homeostasis.

Notably, only one previous study13 studied the expression of lamin A/C in a bone matrix or cartilage. Two studies showed a significant reduction in the formation of a bone matrix in lamin A/C knockout rice14,15. Here it was demonstrated that lamin A/C expression was significantly elevated in elderly patients. This result suggested that lamin A/C expression in the matrix increased with age or spinal activity. The protective role of human activity in IVD tissue likely caused this phenomenon or a type of cell apoptosis, thereby suggesting that the relationship between lamin A/C expression in the nucleus and bone matrix should be further determined by cytological studies.

Lamin A/C is considered to provide stable machinery for the structure of the nuclear membrane. Lamin is an important structure to indirectly or directly communicate gene transcription, the transport of intracellular and extracellular substrates, cell cycle, and differentiation16,17. Destruction of the nucleoprotein lamin structure may lead to nuclear membrane rupture, which can decrease the resistance of cells facing physical pressure or environmental changes, resulting in cell injury or death and a series of pathological changes18. Therefore, the findings of TEM and immunohistochemistry showed that the lamin A/C might be an important molecular barrier to repair IVD degeneration or allow IVD tissues to resist changes in the peripheral environment in different degenerated IVDs.

The present study has several limitations. Firstly, a small number of samples were detected in different degenerative grades of IVDs. Secondly, the expression levels of lamin A/C were only detected at the protein level, not the mRNA level. However, point mutations in lamin A/C can result in serious diseases19. Embryological research20 confirmed the aging of the skeletal system in embryonic development in a zebrafish model with defective lamin A/C. Regenerative medicine data also showed that lamin A/C gene defects caused by zinc matrix metalloproteinase 24 (Zmpste24) deficiency can lead to a decrease in the number and proliferative capacity of stem cells21.

Conclusions

Based on this, the authors speculated that the absence of Zmpste24 or changes in lamin A/C might cause early disc degeneration in young patients. Subsequent work will focus on lamin A/C in early disc degeneration in young patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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