

DISTRIBUTION OF AGGREGAN, COLLAGENS OF I, II, X, XI-ALPHA 2 TYPES OF EXTRACELLULAR MATRIX DURING DEGENERATION OF INTERVERTEBRAL DISC

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ABSTRACT

The article presents the study results of surgical intervertebral disc (L_{IV} - L_V) material during degenerative process. Areas of pulpal core and fibrous ring were analyzed by methods of laser confocal microscopy with markers of aggrecan and collagens of I, II, X, XI alpha-2 types.

Clinical surgical intervertebral disc material was obtained from 7 patients aged 26 to 57 years during scheduled spinal surgeries. Areas of pulpal core and fibrous ring were analyzed. Intervertebral disc tissue fragments were fixed with 4% solution of paraformaldehyde. Studied extracellular matrix proteins were detected by immunohistochemical analysis. The panel of primary antibodies composed the following: mouse antibodies to collagen I [COL-1] (ab90395), rabbit to collagen II (ab34712), mouse to collagen X [COL-10] (ab49945), rabbit to collagen XI alpha-2 (ab196613) and rabbit antibodies to aggrecan [EPR14664] (ab186414). Analyzed specimens were incubated with secondary antibodies to rabbit (ab150080) and mouse (ab150080) IgG, conjugated with fluorescent dye Alexa Fluor® 594, in accordance with species of the used primary antibodies. Analyzed intervertebral disc tissues were stained with Hoechst 33342 dye.

It is established that aggrecan, which occupies the volume of $2.56 \times 10^5 \mu\text{m}^3$ per $1 \times 10^6 \mu\text{m}^3$ of the analyzed tissue, prevails in the extracellular matrix of intervertebral disc. The volume of analyzed areas of type I collagen, occupied by its respective fluorescent signal, amounted to $5.27 \times 10^4 \mu\text{m}^3$ per $1 \times 10^6 \mu\text{m}^3$ of the tissue. Type II collagen was determined by coloration, which was less intense than that of aggrecan, but more intense, than I type collagen: the analyzed volume was $1.21 \times 10^5 \mu\text{m}^3$ per $1 \times 10^6 \mu\text{m}^3$. The volume of fluorescent signal of type X collagen in the studied Z-stacks amounted to $6.09 \times 10^3 \mu\text{m}^3$ per $1 \times 10^6 \mu\text{m}^3$ of intervertebral disc tissue, whereas the fluorescent signal from XI alpha-2 type collagen to $3.40 \times 10^3 \mu\text{m}^3$ per $1 \times 10^6 \mu\text{m}^3$ of the tissue, which is lower than distribution of type I, II and X collagens.

Therefore, the data on the heterogeneity of three-dimensional structures of intervertebral disc, as well as on the spatial distribution of aggrecan and type I, II, X and XI-alpha 2 collagens in intercellular matrix during degenerative changes were obtained. Z-stack intervertebral disc analysis method enables the study of structural components of intercellular matrix in 3D format followed by reconstruction of obtained images both for scientific researches and practical purposes.

KEYWORDS: *aggrecan, collagens of I, II, X, XI-alpha 2 types, intervertebral disc, degenerative process, confocal microscopy.*

INTRODUCTION

Degenerative-dystrophic spine disorders and their surgical treatment currently constitute an im-

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mediate problem in neurosurgery, spinal surgery, traumatology and orthopedics [Konovalov N et al., 2014; Byvaltsev V et al., 2015]. The necessity to develop efficient methods of treatment and prevention predetermines the relevance for detailed description of molecular mechanisms, analysis of structural units in extracellular matrix of an intervertebral disc affected by degenerative changes.

Structural integrity of intervertebral disc tissues is maintained by components of intercellular matrix – collagens produced by chondrocytes, which constitute a group of key cytochemical biomarkers of intervertebral disc pathology [Eyre D, Muir H, 1976; Eyre D et al., 2002; Roughley P, 2004].

The aim of present study was to investigate the range of extracellular matrix components in intervertebral disc (aggrecan and collagens) and specific features of their 3D organization during its degeneration.

MATERIAL AND METHODS

Clinical surgical intervertebral disc (L_{IV} - L_V) material was obtained during scheduled spinal surgeries. The material from 7 patients aged 26 to 57 was studied. Areas of pulpal core and fibrous ring were analyzed. Intervertebral disc tissue fragments were fixed with 4% solution of paraformaldehyde (Sigma-Aldrich, USA) at $+4^\circ\text{C}$ for 24 hours. Specimens were further treated for 1 hour in blocking buffer – Hanks solution (Pan Eco, Russia) with addition of 0.25% of triton X100 (Amresco, USA) and 1% albumin (Amresco, USA). At the next step, the specimens were incubated with primary antibodies at $+4^\circ\text{C}$ for 14 hours. The panel of antibodies (Abcam, USA) composed the following: mouse antibodies to collagen I [COL-1] (ab90395) (1:200), rabbit to collagen II (ab34712), mouse to collagen X [COL-10] (ab49945) (1:200), rabbit to collagen XI alpha-2 (ab196613) (1:200) and rabbit antibodies to aggrecan [EPR14664] (ab186414) (1:200). Analyzed specimens were incubated for 2 hours with secondary antibodies (Abcam, USA) at $+24^\circ\text{C}$ to rabbit (ab150080) and mouse (ab150080) IgG, conjugated with fluorescent dye AlexaFluor® 594 (Life Sciences, USA) in accordance with species of the used primary antibodies. Analyzed intervertebral disc tissues were dyed with Hoechst 33342 (Life Sciences, USA) for 20 minutes. Tissue fragments thus prepared for immunohistochemical analysis were mounted to specimen glass and cast in PROLONG gold (Life Sciences, USA). Prepared specimens were scanned with laser confocal microscope LSM-710 (CarlZeiss, Germany). The study was approved by Ethics Committee of FSBSI Irkutsk Scientific Center of Surgery and Traumatology (Protocol No 10 dated 31.08.2015).

The obtained Z-stacks (optical sections) were analyzed with Imaris 7.2.3 (Bitplane AG, Switzer-

land) software package, which enables 3D reconstruction of morphological images and quantitative evaluation of the analyzed anatomic structures. The software was used to calculate the volumes occupied by fluorescent signal for each type of the studied intercellular matrix components in $1 \times 10^6 \mu\text{m}^3$ of intervertebral disc tissue.

Statistical analysis of the obtained data was performed in Statistica 10 software package using nonparametric methods. Quantitative data are presented as median and quartile range.

RESULTS AND DISCUSSION

Figure 1 presents the range of studied collagen types and aggrecan in tissues of degenerated intervertebral disc – volume of analyzed intervertebral disc areas occupied by the respective fluorescent signal (μm^3) per $1 \times 10^6 \mu\text{m}^3$ of the tissue.

Aggrecan: Aggrecan, which occupies the volume of 2.56×10^5 (2.38×10^5 ; 3.07×10^5) μm^3 per $1 \times 10^6 \mu\text{m}^3$ of the analyzed tissue, prevails in the extracellular matrix of intervertebral disc. The mentioned proteoglycan is known to be the main component of pulpal core [Roughley P et al., 2006]. Areas of parallel-oriented thin-filament structures or thicker structures with dense filament bundles and thickness of 0.2 - $0.4 \mu\text{m}$ were determined. In certain observations, areas with low quantities of aggrecan were found among filamentous aggrecan structures. Meanwhile, aggrecan was distributed either by diffuse localization or it was detected in the form of small (0.35 - $1.5 \mu\text{m}$) lumps, which amounted

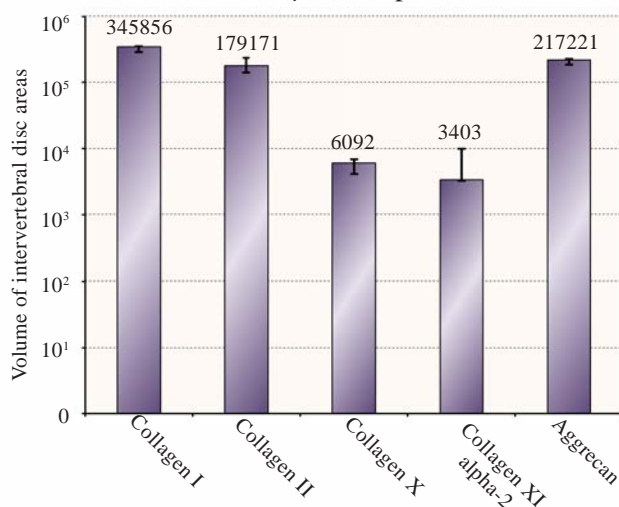


FIGURE 1. Range of collagens and aggrecan in analyzed tissues of degenerated intervertebral disc: volume of analyzed intervertebral disc areas occupied by the fluorescent signal per $1 \times 10^6 \mu\text{m}^3$ of tissue

up to 25% of the analyzed intervertebral disc tissue volume. Cytoplasm in 80% of the cells didn't perceive the dye typical for this marker. Zones of absence of this proteoglycan in matrix were identified only in 5% of observations. However, these zones contained individual groups of cells with intense synthesis of aggrecan (Fig. 2).

Type I collagen: Type I collagen was characterized by less intense coloration than aggrecan: volume of analyzed intervertebral disc areas occupied by fluorescent signal amounted to 5.27×10^4

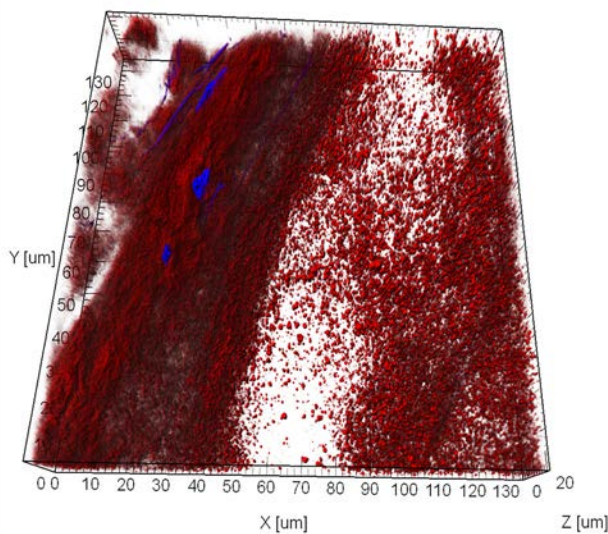


FIGURE 2. Specific features of aggrecan structure in intervertebral disc tissue during its degeneration

(1.29×10^4 ; 2.30×10^5) μm^3 per $1 \times 10^6 \mu\text{m}^3$ of the tissue. Zones with parallel or multidirectional orientation of thin-filament organized structures were identified. Areas with diffuse or lump-shaped distribution of dye were found in 70% of analyzed Z-stacks in comparison with aggrecan. Zones with low content of type I collagen were found, which amounted to 40% of Z-stacks compared to similar aggrecan distribution zones (Fig. 3A).

Type II collagen: Type II collagen was also identified by coloration, which was less intense than that of aggrecan, but more intense than type I collagen: volume of intervertebral disc areas occupied by fluorescent signal amounted to 1.21×10^5 (1.05×10^5 ; 1.60×10^5) μm^3 per $1 \times 10^6 \mu\text{m}^3$ of intervertebral disc tissue. In terms of type I collagen distribution the analyzed zones were identified as zones of similar thin-filament structure, but having more multidirectional orientation. Areas of Z-stacks with diffuse or lump-shaped dye distributions were also identified (Fig. 3B).

It should be noted that large quantities of active cells synthesizing type I and II collagens were observed in the studied areas of Z-stacks with low content of these proteins, which apparently reflects the course of different phases of intervertebral disc tissue remodeling process targeted at local restoration of intercellular matrix.

Type X collagen: Type X collagen was also characterized by less intense staining than aggrecan, and occurred mainly in the cytoplasm of cells within the analyzed samples of intervertebral disc tissue. The volume of fluorescent signal produced by type X collagen in Z-stacks amounted to 6.09×10^3 (4.15×10^3 ; 6.87×10^3) μm^3 per $1 \times 10^6 \mu\text{m}^3$ of intervertebral disc tissue. This protein is known to be absent under normal conditions, and its structural characteristics is typical for degenerative intervertebral disc process, which corresponds to obtained data [Boos N et al., 1997] (Fig. 3C). It is known that the production of type X collagen is predominantly typical for cartilage tissue hypertrophy process. It has been shown that when chondrocytes lose the ability to produce II, VI, IX, XI type collagen and proteoglycans, they activate synthesis of type X collagen [Boos N et al., 1997].

Collagen type XI alpha-2: Collagen type XI alpha-2 was identified in intervertebral disc of one patient within the entire observation series. The volume occupied by the fluorescent signal of type XI alpha-2 collagen in Z-stacks of analyzed intervertebral disc areas amounted to 3.40×10^3

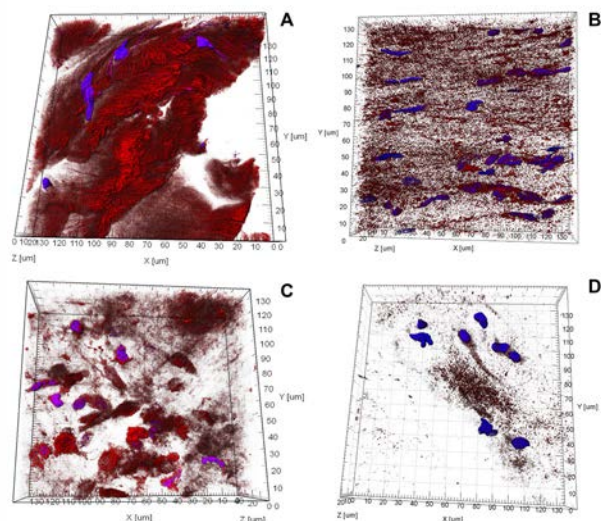


FIGURE 3. Distribution of collagens in intercellular matrix of intervertebral disc during its degeneration Spatial structure of collagen I (A) and II (B); synthesis of collagen X (C) and XI alpha-2 (D) by chondrocytes

(3.27×10^3 ; 1.00×10^4) μm^3 per $1 \times 10^6 \mu\text{m}^3$ of tissue, which is lower than distribution of I, II and X type collagens. Collagen of XI alpha-2 type was predominantly non-identifiable in the main mass of disc tissue, and was determined mainly in diffusely colored zones of extracellular matrix, containing cell aggregations (Fig. 3D).

CONCLUSION

Present study was aimed to confirm heterogeneity of 3D structures of intervertebral disc, as well as spatial distribution of aggrecan and collagens of I, II, X and XI types in intercellular matrix under the conditions developed destructive degenerative processes of intervertebral disc. It has been

established that aggrecan is characterized by diffuse spatial distribution compared to collagens. Type II collagen was characterized by less intense coloration, than that of aggrecan, but more intense than type I collagen. The volume of fluorescent signal of type X and XI alpha-2 collagen was lower than that of type I, II collagens, whereby these proteins were mainly localized inside the cells.

Therefore, analysis of Z-stacks of the intervertebral disc using laser confocal microscopy allows to objectively evaluate 3D analysis of structural components in intercellular matrix of intervertebral disc followed by reconstruction of the obtained images, which may be used in developing prospective diagnostic or treatment technologies.

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