World Applied Sciences Journal 32 (6): 1039-1044, 2014
ISSN 1818-4952

DOI: 10.5829/idosi.wasj.2014.32.06.790

Changes in the Intervertebral Discs Annulus Fibrosus at Staph Infection Modeling

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Abstract: Long-term infection could affect other organs and tissues. In the present study, we investigated the influence of tibial osteomyelitis caused by Staphylococcus aureus on intervertebral discs annulus fibrosus in Wistar rats. Histochemical assay was carried out on sulfated glycosaminoglycans and neutral glycoproteins of annulus fibrosus. Immunochemical method was applied to I and II type collagen, fibronectin, fibulin-2 and matrilin-2. The role of persisting staphylococcal infection in the initiation and development of degenerative changes of the fibrous cartilage of intervertebral discs was demonstrated. Changes in the extracellular matrix components of annulus fibrosus were revealed 1 month after Staphylococcus aureus inoculation. Progressive disorders in sulfated glycosaminoglycans metabolism accompanied by changes of collagen type predominance replacement suggest fibrous tranformation in intervertebral discs. Increase of neutral glycoproteins due to separate fractions, in particular, fibulin-2 could be considered as compensatory reaction on progressing overpatchings of fibrocartilage extracellular matrix components. Reorganizations mentioned are supposed to promote further dystrophic-degenerative changes in intervertebral discs.

Key word: Intervertebral discs · Annulus fibrosus · Staphylococcus aureus · Collagen · Glycosaminoglycans · Neutral glycoproteins · Fibronectin · Fibulin-2 · Matrilin-2

INTRODUCTION

Progress in antimicrobial therapy methods developing is undoubted however staphylococcal infections for over 50 years are still supposed to be at the bottom of morbidity and mortality. Staphylococcus aureus in the United States each year causes more cases of infectious diseases than tuberculosis, viral hepatitis and AIDS combined and methicillin-resistant strains are especially deleterious [1, 2]. Evident symptoms of infection are highly focused, while minor manifestations of macro- and microorganisms interaction are not less important. In particular, it was shown a direct relationship between the focal persisting bacterial infection and the development of degenerative-dystrophic changes in rabbit intervertebral discs [3]. Considerably those pathological changes in the intervertebral disks could manifest the syndrome of degenerative-dystrophic changes in mesenchymal derivatives during local chronic inflammatory process [4]. In the present study, we investigated the influence of tibial osteomyelitis caused by Staphylococcus aureus on the ratio of annulus fibrosus extracellular matrix components in rats.

MATERIALS AND METHODS

Study Design: The experiment was performed in twenty four male Wistar rats (180-220 g, 2,5 months old). All animals were treated according to protocols approved by the animal care institutional review board. Eighteen rats were subjected the experimental tibial myelitis. Using sterile surgical conditions shin-bone trepanation was carried out under halothane anesthesia, hole was plugged with cotton thread containing Staphylococcus aureus, strain 209 (10^7 cfu). Animals were decapitated 1, 2 and 3-months after surgery. Six intact rats were used as a control. Six animals with the trephined tibia followed by the introduction of sterile cotton thread were used as an additional control.

Immunohistochemistry: Specimens (tail intervertebral disks) were fixed in 12% formalin followed by dehydratation in ethanol and embedding in paraffin. One section was used for the conventional hematoxylin and eosin method. Collagen fibers were stained by Van Gieson's picrofuchsin, sulfated glycosaminoglycans (SGAGs) – by alcian blue (pH 1,0) and neutral

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glycoproteins – by McManus PAS reaction [5]. The extracellular matrix parameters were estimated by immunocytochemistry based on indirect streptavidin biotin peroxidase method as described previously [6, 7] according to the manufacturer's instructions. Triton X-100 (0,1%) was used for antigen demasking procedure for 5 min. Deparaffinized sections were incubated with primary antibodies: Anti-Collagen Type I (COL-1, Mouse IgG, Santa Cruz Inc.), Anti-COL2A1 (M2139, Mouse IgG2, Santa Cruz Inc.), Anti-Fibronectin (Isotype: Mouse IgG1, Clone: IST-9, Santa Cruz Inc.), Anti-Fibulin-2 (H-250, rabbit polyclonal, Santa Cruz Inc.) and Anti-Matrilin-2 (H-65, rabbit polyclonal, Santa Cruz Inc.). All incubations were performed for 60 min at room temperature. Immunostaining was performed using Novocastra Peroxidase Detection System (Ready-to-Use) kit (Code No. RE7110-K), which employed the streptavidin-biotin technique and DAB Substrate/Chromogen System for visualization.

Image Analysis: Sections were viewed by light microscopy (area: 64500 mkm^2 [8], magnification: ×400 per each experimental group). Staining intensity and integrated density were analyzed quantitatively using Image J 1.42g software (National institutes of Health, USA). RGB channels were applied to reveal tinctorial characteristics of collagen fibers and neutral glycoproteins (Red) and SGAGs (Blue).

Data Analysis: The results were performed as a percentage obtained by the following relationship:% structure = S_s/S_t, S_s is the stained fibroblasts area and S_t is the total investigated area. Results were expressed as the mean (± SEM). Statistical analyses were performed using Kruskal-Wallis H test and Mann-Whitney U test with Bonferroni correction. Statistical significant was accepted at $p \leq 0.05$.

RESULTS

The localized osteomyelitis model we analysed led to changes in rat health status (fever, appetite loss). After 1 month from the date of inoculation Staphylococcus aureus necrosis of bone marrow, productive inflammatory process in endosteum and periosteum, osteoclast-mediated bone resorption. Later on, in 2 and 3 months after inoculation of Staphylococcus aureus bone marrow offered granulation tissue fields bounded the necrosis foci. Bone trabeculae in spongy bone tissue were fragmented, few osteocytes were located irregularly. Focal dispersions could be visualized in bone matrix.

1 month after staphylococcal infection nidus has been produced in the tibia statistically significant decrease of staining intensity and integrated density of sulfated glycosaminoglycans (SGAGs) were revealed despite relative area of SGAGs was not changed (Table 1). Findings are consistent with the data [9, 10] that the changes of glycosaminoglycans included in proteoglycans annulus fibrosus are the most permanent sign of dystrophic-degenerative changes of intervertebral discs. Moreover, changes in tinctorial properties of neutral glycoproteins observed 1 month after Staphylococcus inoculation may indicates changes in the ratio of different neutral glycoproteins fractions that is confirmed by immunohistochemical analysis (Table 1).

Table 1: Histochemical analysis of extracellular matrix of intervertebral discs (annulus fibrosus), M±m

		Inflammation		
	Control	1 month	2 month	3 month
Collagen fibers				
AR	78,55±1,06	75,84±0,82	73,61±0,70*	66,73±1,08*
SI	90,29±1,17	85,53±1,88	84,39±1,60*	80,95±2,39*
ID	7094,7±130,4	6451,2±128,3*	6209,9±131,0*	5337,1±138,1*
Red	187,91±1,03	187,38±0,94	173,63±0,95*	151,21±1,01*
Neutral glycoproteins				
AR	63,38±1,31	64,82±1,31	68,94±0,93*	73,04±0,91*
SI	16,02±1,10	15,63±0,65	19,31±0,87*	23,85±2,69
ID	1042,6±79,3	1013,1±45,5	1332,6±63,5*	1785,6±206,9*
Red	195,43±1,85	188,15±0,97*	184,44±1,12*	169,16±2,35*
Sulfated glycosaminoglycans				
AR	81,73±1,17	81,19±0,90	63,48±1,57*	40,01±2,32*
SI	63,95±2,26	49,67±2,85*	43,79±2,49*	27,55±1,52*
ID	5208,1±184,4	4074,9±233,4*	2766,5±171,4*	1069,5±79,7*
Blue	187,38±0,94	181,87±1,82	178,97±1,22*	151,90±1,07*

* - p<0,05 compared to control

Table 2: Immunohistochemical analysis of extracellular matrix of intervertebral discs (annulus fibrosus), M±m

		Inflammation		
	Control	1 month	2 month	3 month
Collagen fibers I type				
AR	80,09±0,63	84,12±0,74*	88,16±0,54*	76,79±1,65
SI	28,38±1,34	26,63±1,57	7,84±0,49*	22,48±1,33*
ID	2264,5±103,1	2230,0±131,9	690,7±43,6*	1663,6±83,6*
Collagen fibers II type				
AR	89,32±1,24	67,10±0,83*	65,26±0,99*	77,34±0,80*
SI	8,69±0,88	50,70±0,67*	54,57±0,88*	51,27±0,57*
ID	788,1±82,8	3399,0±58,8*	3547,9±65,7*	3976,8±72,8*
Fibronectine				
AR	73,11±0,81	78,70±0,69*	72,24±0,90	65,56±0,81*
SI	38,09±1,52	42,13±1,37	17,18±0,75*	17,03±1,27*
ID	2805,6±127,0	3298,9±102,4*	1247,2±58,5*	1130,1±85,4*
Fibulin-2				
AR	67,02±1,11	67,75±1,11	83,22±0,92*	82,20±1,08*
SI	21,60±1,14	34,06±1,14	54,63±1,61*	77,34±1,41*
ID	1442,0±80,5	2301,9±85,0*	4522,6±131,3*	6373,3±164,3*
Matrilin-2				
AR	56,28±2,36	68,29±0,63*	69,75±0,84*	63,69±1,47*
SI	9,54±1,28	9,99±0,22	11,23±0,13*	26,20±1,57*
ID	437,4±51,8	681,4±15,6*	782,5±11,2*	1664,5±106,8*

* - $p<0,05$ compared to control

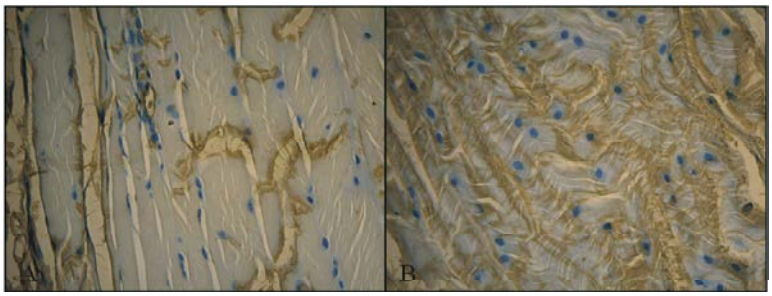

Fig. 1: Fragment of rat annulus fibrosus of the intervertebral disk. Increased content of fibulin-2. (A) 2 month after S. aureus inoculation. (B) 3 month after S. aureus inoculation. Immunostaining for fibulin-2, Ehrlich's hematoxylin. × 400.

The ratio of integrated density of several markers (fibronectin/fibulin-2/matrilin-2) was 4,8/3,8/1 compared to the control (6,4/3,8/1). This reflects the increase of fibronectin fraction in amorphous substance of the annulus fibrosus 1 month after staphylococcal infection. Taking into account the important role of this glycoprotein collagen forming the possibility of collagen type I increase demonstrated becomes clear (Table 2).

2 months after inoculation of Staphylococcus aureus in the tibia we observed changes in tinctorial properties, staining intensity and integrated density of collagen fibers and neutral glycoproteins of extracellular matrix (Table 1) as a result of alterations in neutral glycoproteins composition (fibronectin/fibulin-2/matrilin-2 ratio was 1,7/6,3/1) (Fig. 1, A) and collagen predominance (collagen I/collagen II ratio was 1/5,1 compared to control (2,9/1). Fibronectin integrated density reduced by 2,6 times was accompanied by collagen I type integrated density decrease and collagen II type integrated density increase (Table 2, Fig. 2, A). Moreover, relative area of SGAGs was significally lower by 1,3 times (Fig. 3, A). Findings suggest fibrotic changes in annulus fibrosus advanced 2 month after surgery that is noteworthy at studing of degenerative processes in the intervertebral discs [11].

3 months after bacterial infection nidus creation in the tibia indices collagen fibers relative area went down progressively ($p<0,05$) and SGAGs relative area was increased (Fig. 3, B). Violations of the SGAGs metabolism and consequently proteoglycans at persisting staphylococcal infection are likely reflected on

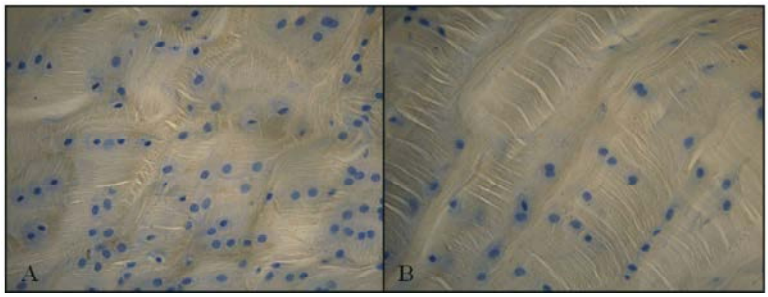

Fig. 2: Fragment of rat annulus fibrosus of the intervertebral disk. Reduced content of collagen type II in lamellae. (A) 2 month after S. aureus inoculation. (B) 3 month after S. aureus inoculation. Immunostaining for collagen type II, Ehrlich's hematoxylin. × 400.

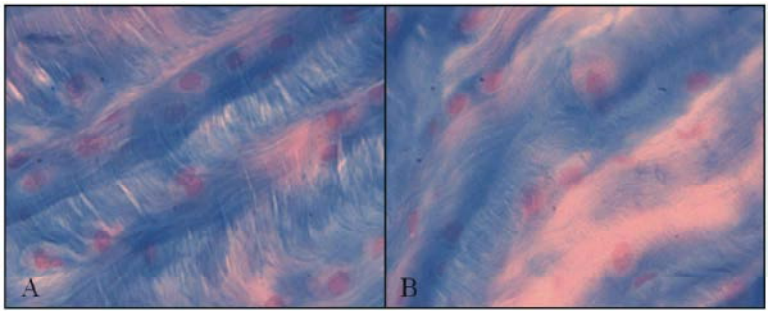

Fig. 3: Fragment of rat annulus fibrosus of the intervertebral disk. (A) Reduced content of sulfated glycosaminoglycans 2 month after S. aureus inoculation. (B) Increase of chondrocyte polymorphism, reduced sulfated glycosaminoglycans area 3 month after S. aureus inoculation. Combined Alcian blue (pH 1.0) and Mayer's carmalum staining. × 400.

viscoelastic characteristics of intervertebral discs [12]. Moreover, it was noted further growth neutral glycoproteins area together with persistent shift in the fractions ratio (Table 1).

In complex with fibrous component changes (Fig. 2, B) and neutral glycoproteins of extracellular matrix (Fig. 1, B), additional biomechanical loadings could trigger hernia formation. Reduced SGAGs area was revealed (Fig. 3, B).

These data confirm clinical observations of heavy manifestations of intervertebral disks degenerative diseases and demonstrate adverse outcome in the treatment of patients with chronic inflammatory nidi [4].

DISCUSSION

In recent years more attention is paid to restoration of functioning tissues by cell therapy tissue engineering methods at intervertebral discs degenerative processes [13]. Knowledge of pathophysiological processes in target tissues, in particular, at chronic staph infection is applied to develop tissue engineering strategies. Cells of annulus fibrosus manifest chondrocyte phenotype with prevalent expression of collagen II type [14-16].

Increase of vascularization of external departments of the annulus revealed in the presence of bacterial infection nidus could possibly affect the rate of metabolic processes in intervertebral disks. It is necessary to notice, the experimental data demonstrated shows the increase of collagen type II integrated density, as well as the polarization ratio examined types of collagen in favor of collagen type II that may indicate the possibility of fibrous changes in the annulus fibrosus during bacterial infection [11]. Notable for the fact that changes in the collagen type I and collagen type II integrated density observed together with changes in the fibronectin expression (Table 2).

Fibronectin and integrins are supposed to play an important role in collagen fibril forming [17]. The requirement of fibronectin for collagen fibril assembly is not restricted to fibroblasts. Collagen fibril assembly by vascular smooth muscle cells was inhibited by an anti-a2b1 integrin antibody and accelerated by an a2b1 integrin antibody that stimulates a high-affinity binding state of the integrin [18]. In the same study, newly assembled collagen fibrils were found to colocalize with newly assembled fibronectin fibrils. Also, the inhibition of fibronectin assembly with an anti-a5b1 integrin antibody

completely inhibited collagen assembly. It seems probable, therefore, that fibronectin fibril assembly and collagen fibril assembly have mechanistic elements in common, involving functional integration of the cytoskeleton with plasma membrane-located integrins. In the case of fibronectin, an a5b1 integrin-induced conformational change is necessary to promote fibrillogenesis. It is less clear how integrins and fibronectin catalyze collagen fibrillogenesis. A tantalizing possibility is that fibronectin and/or integrins induce a conformational change in collagen to accelerate fibrillogenesis [17].

The changes of the annulus fibrosus extracellular matrix mentioned combined with a progressive decline in relative area and staining intensity of SGAGs. Most likely violations of SGAGs and, therefore, proteoglycans, are reflected on the elastic and viscous properties of intervertebral disks at long-term staph infection [12].

CONCLUSION

Initiation and restructurization of annulus fibrosus extracellular matrix was demonstrated in the model of tibial osteomyelitis caused by Staphylococcus aureus (strain 209) in Wistar rats. Progressive decrease in the content of sulfated glycosaminoglycans, significant reduction of collagen fibers relative area accompanied by changes in the ratio collagen type I/II, compensatory increase of neutral glycoproteins and shifting balance of separate fractions were demonstrated. These changes of extracellular matrix fibrous cartilage of intervertebral discs could alter nonvascular microcirculation ways, prelymphatics that should be considered in the treatment and prevention of osteochondrosis.

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