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Comparative Immunohistochemical Study on Collagen Types in Kidney during Aging and Hypertension

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Renal fibrosis is characterized by increased synthesis of collagen molecules in the renal parenchyma and interstitium. In the present study, we demonstrated the expression of collagen types I and V and procollagen type III in the renal structure of 12-month-old spontaneously hypertensive rats and age-matched Wistar rats. The main findings included higher immunoreactivity of the examined molecules under hypertensive conditions. The results were obtained by semi-quantitative analysis of the immunohistochemical expression. We found pronounced intraglomerular expansion of collagen type III and V in spontaneously hypertensive rats compared to the normotensive group. In addition, the accumulation of collagen fibers in the renal parenchyma and interstitium was also represented by Mallory's trichrome method. We analyzed the severity of the renal fibrosis as a result of aging as well as in a case of essential hypertension. In conclusion, the development of renal fibrosis is more severe under hypertensive conditions and is associated with specific distribution of the analyzed collagen types.

*Key words:*collagen, kidney, hypertension, aging

Introduction

Renal fibrosis is a nonspecific process, which can be observed as a result of aging, as well as various pathological conditions. In addition, hypertensive kidney damage is accompanied by pronounced expansion of extracellular molecules both in the renal cortex and medulla [8]. It seems that the severity of interstitial fibrosis rather than the glomerular changes correlates better with the renal functional capacity [19]. The development of renal fibrosis depends on the activity of renal interstitial fibroblasts, inflammatory cells, cytokines and matrix metalloproteinases [4]. The accumulation of collagen fibers can be represented by different histological techniques, which include Masson's trichrome method, Sirius Red, which are strongly specific for collagen types I and III, as well as immunohistochemical methods [17]. Many studies reveal that the fibrous skeleton of the kidney contains various collagen molecules with specific distribution in the renal structure. It is well known that collagen types I, III and V are all expressed in the renal interstitium [6]. Collagen types I and III are not found in the glomeruli under physiological conditions, which suggests that the altered expression of these molecules may serve as indicator of glomerular injury [6, 7]. Collagen type V is

also found in the glomerular and extraglomerular mesangial matrix [6]. Renal fibrosis is usually associated with increased synthesis of collagen types I and III, where cellular phenotypic changes may play a crucial role in the process [4]. On the other hand, there is insufficient information regarding the changes in the expression of collagen type V during the development of renal fibrosis due to hypertensive kidney damage.

The spontaneously hypertensive rat (SHR) is a widely used model for essential hypertension. In this strain, the hypertensive-induced renal morphological alterations correlate well with these observed in the human population [10].

The aim of the current study was to demonstrate the hypertension-induced renal fibrosis in SHR and to establish the distribution of collagen types I and V and procollagen III in the renal structure. We have also shown the altered expression of these molecules in cases of hypertensive and age-related renal fibrosis.

Materials and Methods

Experimental animals and tissue preparation

Male SHR and Wistar rats (WR), available at the Medical University of Sofia, aged 12 months (n=3; per group) were used for this study with the approval of the University Committee on Animal Resources. The rats were anesthetized intraperitoneally with Thiopental 40 mg/kg b.w. The chest cavity was opened and transcardial perfusion was made with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. The kidneys were quickly removed and fixed in 10% neutral buffered formalin. After routine paraffin embedding, 5 μm thick sections were cut and stained with Mallory's trichrome method. The paraffin was removed, after which the slides were placed in 0.1% fuchsin for 1-2 minutes, washed and placed in 1% solution of phosphomolybdic acid for 3-5 minutes. After thorough rinsing, the slides were placed in a mixture of aniline blue, orange G and oxalic acid for 2 minutes, washed again and embedded in entellan.

Immunohistochemistry

For the immunohistochemical analysis, the 5 μm-thick sections mounted on gelatin-coated slides were preincubated for one hour in 5% normal goat serum. After that, incubation with a primary antibody was done for 24 h at room temperature. The following reagents were used: mouse monoclonal anti-collagen type I IgG antibody (Santa Cruz Biotechnology Catalogue No. sc-293182, Santa Cruz Biotechnology, Inc., Heidelberg, Germany); mouse monoclonal anti-procollagen type III IgG antibody (Santa Cruz Biotechnology Catalogue No. sc-166316); mouse monoclonal anti-collagen type V IgG antibody (Santa Cruz Biotechnology Catalogue No. sc-166155); all antibodies were used at concentration 1:500. After rinsing in PBS, incubation in biotinylated goat anti-mouse IgG antibody at concentration 1:500 for two hours was performed. The sections were washed in PBS and incubated in avidin-biotin peroxidase complex for 1 h. This step was followed by rinsing in PBS and then in 0.05 M Tris−HCl buffer, pH 7.6, which preceded incubation in 0.05% 3,3-diaminobenzidine (DAB) containing 1% hydrogen peroxide (H_2O_2) (1:100) for visualization of the reaction. Sections were briefly washed in 0.05 M Tris−HCl buffer, pH 7.6. The slides were air-dried for 24 h, then rinsed in distilled water for five minutes, three times, contrastained with hematoxylin, air-dried again and coverslipped with Entellan. One section per animal from the kidney (a total of twelve sections) were used as controls. All were incubated in the way previously described, but omitting the primary or secondary antibody. All controls were negative.

Semi-quantitative analysis of the immunohistochemical expression

For semi-quantitative analysis of the expression of collagen types I and V and procollagen type III, we used software ImageJ 1.52a, freely downloaded from the website of the National Institute of Health (NIH) (http://imagej.nih.gov/ij/). The intensity of staining was assessed through the IHC Profiler plugin, freely downloaded from the Sourceforge website (https://sourceforge.net/projects/ihcprofiler/). The IHC Profiler assigned a score to each visual field in a four tier system – high positive (3+), positive (2+), low positive (1+) and negative (0). Five slides were used from each organ. We analyzed at least ten randomly selected visual fields on each slide. The final score was the average of the scores of all visual fields as calculated by the IHC Profiler.

Results

Histological findings

In 12-month-old WR, we found moderate expansion of collagen fibers among the renal parenchyma and interstitial connective tissue, stained in blue color by Mallory's trichrome method. In the renal cortex, accumulations of collagen fibers were described in the region of the parietal layer of the glomerular capsule and glomerular capillary tufts of the three main types of nephrons. Perivascular fibrosis was represented by concentric layers of collagen fibers around interlobular and intralobular blood vessels. Interstitial fibrosis was established among various sections of proximal and distal tubules (**Fig. 1**).

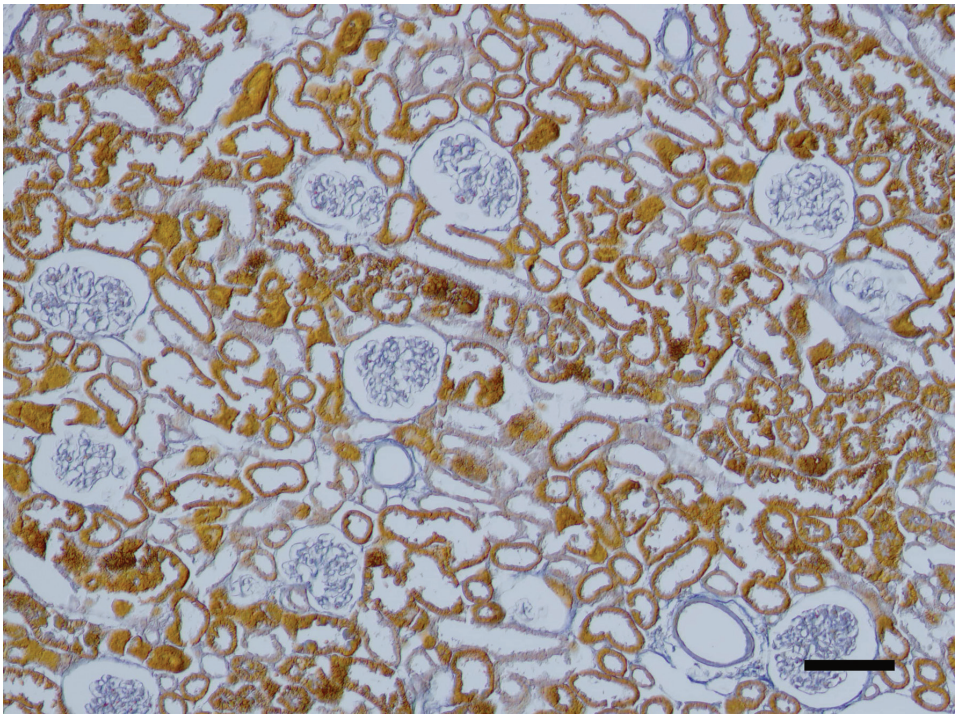

Fig. 1. Photomicrograph of kidney stained with Mallory's trichrome method. WR, age – 12 months. Scale bar – 150 μm.

In 12-month-old SHR, we established more pronounced expansion of collagen fibers in the interstitial connective tissue and the structural elements of the renal corpuscles in SHR compared to the normotensive rats. The renal fibrosis was more severe in the inner cortex and medulla in the hypertensive rats. Mallory's trichrome method revealed extensive areas of collagen accumulations in the region of the parietal layer of the glomerular capsule and glomerular capillary tufts of the midcortical and juxtamedullary nephrons as well as around the tubular epithelial cells. The described morphological alterations showed characteristics of advanced glomerulosclerosis and tubular atrophy. We didn't find such selectivity of the renal injury in WR (**Fig. 2**).

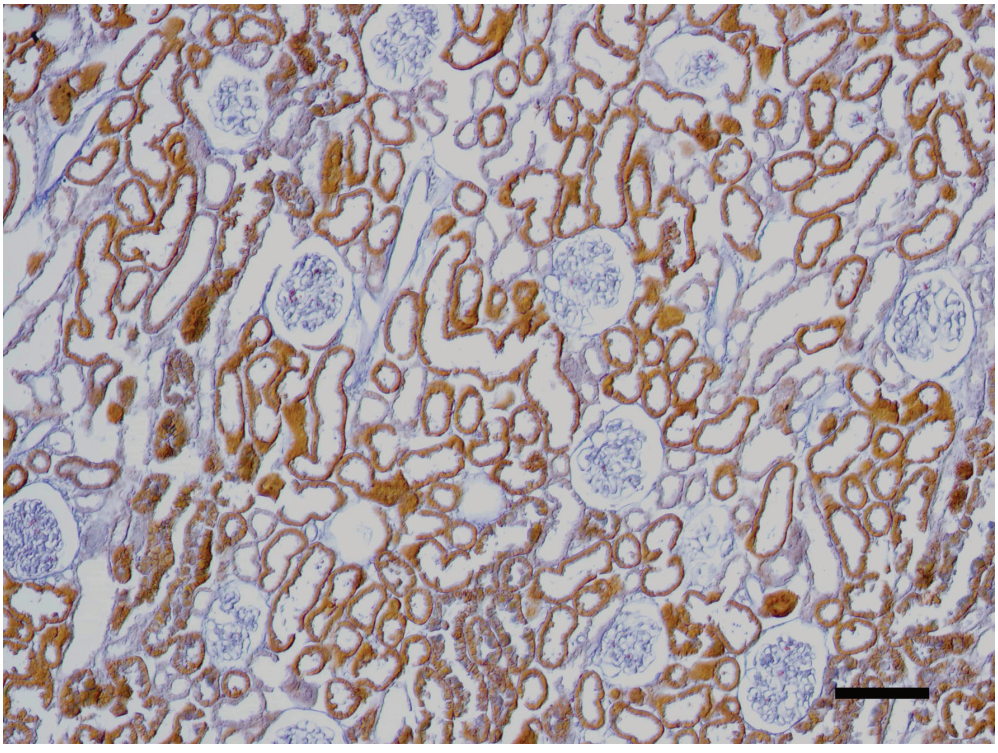

Fig. 2. Photomicrograph of kidney stained with Mallory's trichrome method. SHR, age – 12 months. Scale bar – 100 μm.

Expression of collagen type I and V and procollagen type III in the kidney of WR

In 12-month-old WR, we described heterogeneous distribution of the collagen types I and V and procollagen type III. Collagen type I was found along the proximal and distal convoluted tubules and moderate expression in the renal corpuscles of the three types of nephrons. Collagen type V showed high positive reaction in the glomeruli as well as the interstitial connective tissue. Procollagen type III was found mainly in the interstitial connective tissue and low positive reaction in the renal corpuscles (**Fig. 3A, 3B, 3C**).

Expression of collagen type I and V and procollagen type III in the kidney of SHR

The immunohistochemical study on the distribution of collagen types I and V and procollagen type III in the renal structure showed higher expression of the examined molecules in SHR. The results showed that the progression of the renal fibrosis under

hypertension is associated with increased expansion mainly of collagen type V and procollagen type III. We found increased proportion of intraglomerular distribution of collagen type V and procollagen type III in SHR compared to the normotensive rats. In WR, the renal medulla showed low positive to positive immunoreactivity for collagen type I and procollagen type III, while in SHR quite positive reaction for collagen type I and V was established (**Fig. 3D, 3E, 3F**).

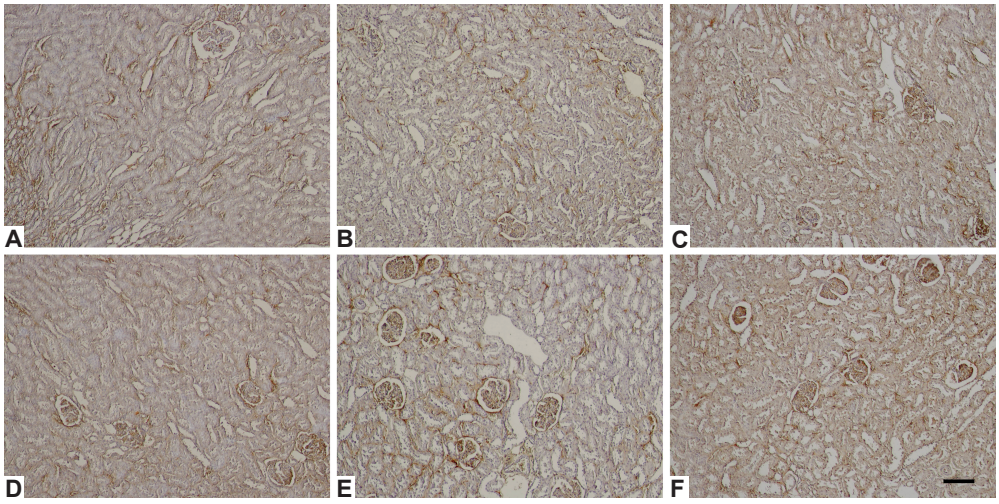

Fig. 3. Immunohistochemical expression of collagen type I and V and procollagen type III in the kidney of WR and SHR. **A**. Collagen type I in 12-month-old WR; **B.** Procollagen type III in 12-month-old WR; **C**. Collagen type V in 12-month-old WR; **D**. Collagen type I in 12-month-old SHR. **E**. Procollagen type III in 12-month-old SHR; **F.** Collagen type V in 12-month-old SHR. Scale bar – 100 μm.

Semi-quantitative analysis

The intensity of the immunohistochemical reaction varied between the kidney of SHR and WR and between the studied types of collagen and procollagen type III. In order to objectify these findings, we calculated the expression semi-quantitatively using the IHC Profiler. Results are summarized in **Table 1**.

Table 1. Semi-quantitative analysis of the immunohistochemical expression of collagen type I and V and procollagen type III in kidney of SHR and WR. The percentage for each score represents the percentage of visual fields that the IHC Profiler assigned this score to.

Type of collagen/procollagen	Kidney	
	SHR	WR
Collagen type I	Positive (2+) (48%)	Positive (2+) (33%)
	Low-positive (1+) (34%)	Low-positive (1+) (31%)
	Negative (0) (18%)	Negative (0) (36%)
Procollagen type III	High-positive (3+) (63%)	High-positive (3+) (49%)
	Positive (2+) (21%)	Positive (2+) (31%)
	Low-positive (1+) (16%)	Low-positive (1+) (20%)
Collagen type V	High-positive (3+) (57%)	High-positive (3+) (41%)
	Positive (2+) (21%)	Positive (2+) (34%)
	Low-positive (1+) (22%)	Low-positive (1+) (25%)

Discussion

It has been proved beyond doubt that the prolonged and untreated essential hypertension is a primary risk factor for chronic kidney disease [16]. Our results show that renal fibrosis in SHR compared to normotensive controls is associated with increased expression of the examined collagen molecules, mainly collagen types III and V. A number of authors have shown that the progression of renal fibrotic changes is usually associated with accumulation of collagen types I and III [1, 4]. In addition, we established a significantly higher expression of collagen type V in SHR, which suggests that this molecule may play a key role in the development of hypertension-induced renal fibrosis. The elevated blood pressure causes glomerular injury and leads to increased number of sclerotic glomeruli [5]. Glomerulosclerosis is characterized by accumulation of collagen types I and III in the glomerular capillary tufts, which is demonstrated in the present study. As a structural element of the glomerular mesangium, collagen type V showed positive reaction in the region of the renal corpuscles in SHR and WR. The immunohistochemical expression for collagen type V was higher in SHR compared to normotensive controls, which indicates that this extracellular molecule is also involved in the development of the glomerular damage. On the other hand, there is contradictory evidence regarding the effect of interstitial fibrosis on the development of renal morphological changes in the structural elements of the nephrons. Some authors have demonstrated intact tubular segments of the nephrons, which are surrounded by an extensive mass of collagen fibers and areas of tubular degeneration [13, 14]. However, the influence of prolonged interstitial fibrosis as a possible harmful factor for chronic kidney damage has not been excluded [13, 14].

In the present study, we also found that the expansion of collagen molecules in SHR is more pronounced in the inner rather than the outer cortex. Some authors have tried to explain the described selectivity of renal alterations with the higher glomerular capillary pressure of midcortical and juxtamedullary nephrons under hypertensive conditions [11]. On the other hand, the aging kidney is characterized by numerous inevitable morphological changes, similar to those observed in essential hypertension, such as tubular atrophy, glomerulosclerosis and tubulointerstitial fibrosis [3]. Unfortunately, the lack of strongly specific alterations makes it difficult to determine the etiology of the kidney damage. Jenkins et al. reported periglomerular fibrosis in glomeruli characterized by open capillary loops and the presence of concentric layers of collagen fibers in the region of the basement membrane of glomerular capsule. Moreover, the authors termed the expansion of interstitial collagen as renal 'fibrosis', and the increase of the structural elements of the basement membrane as 'sclerosis' [12]. It is well known that collagen type IV is mainly found in the basement membranes. In our study, the higher expression of collagen type V in SHR in the parietal and visceral layer of glomerular capsule may contribute to the progression of sclerosis. In rat kidney, the complete degeneration of a single nephron is accompanied by $40 \times 10^6\ \mu m^3$ lack of epithelial tissue, which is replaced by extracellular fibers [18].

Indeed, the development of renal interstitial fibrosis is a multifactorial process, which is closely associated with the increased activity of various cell types. It is well known that renal interstitial fibroblasts play a key role in the synthesis of extracellular molecules. However, the examination of these cells is usually difficult, because of the lack of strongly specific expression of marker molecules [2]. The demarcation of fibroblasts from other interstitial cells is based on the presence of F-actin in their cytoskeleton and a well-developed rough endpolasmatic reticulum [13]. Many studies have suggested the primary role of myofibroblasts in the course of hypertensive induced renal fibrosis. There are several hypotheses for the origin of these cells, including from

interstitial fibroblasts [4]. It seems that interstitial dendritic cells are also involved in the development of renal injury, because some authors have demonstrated a positive correlation between the abundance of these cells and the tubulointerstitial changes [9]. As aging progresses, the tubular epithelial cells may acquire secretory phenotype properties, which are associated with increased production of proinflammatory cytokines [20]. Morphometric studies have demonstrated the evaluation of renal fibrosis on slides stained by trichrome and immunohistochemical methods [15]. Unfortunately, these methods have some limitations concerning the renal biopsy technique.

Conclusion

In conclusion, hypertensive kidney damage is associated with more severe morphological changes in the renal parenchyma and interstitium compared to the physiologically aging kidney. Renal fibrosis is characterized by an increased synthesis of collagen types I and III, as confirmed by our results. The observed high positive immunoreactivity for collagen type V in the present study shows that this molecule likely also plays a crucial role in this process.

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