

EXPERIMENTAL STUDY

Differential collagen expression in kidney and heart during hypertension

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ABSTRACT

AIM: The aim of the present study was to investigate the immunohistochemical expression of selected collagen types, namely collagen types I and V and procollagen type III in the renal parenchyma and interstitium and in the myocardium of spontaneously hypertensive rats.

MATERIAL AND METHODS: For the present study, we used two age groups of 6- and 12-month-old spontaneously hypertensive rats. An immunohistochemical analysis was conducted with monoclonal antibodies against collagen types I and V and procollagen type III. A semi-quantitative analysis of immunostaining intensity was conducted with the Image J software.

RESULTS: In the kidney, all three molecules showed higher expression at the age of 12 months, which was particularly notable for procollagen type III and collagen type V, which stained as highly-positive. In the myocardium, the immunoreactivity of collagen types I and V was stronger in 12-month-old animals, while that of procollagen type III did not change substantially.

CONCLUSION: The present study suggests a role of collagen types III and V in hypertensive kidney disease, while also establishing the role of increased expression of collagen types I and V in adverse myocardial remodeling (Tab. 1, Fig. 2, Ref. 48). Text in PDF www.elis.sk.

KEY WORDS: collagen, kidney, myocardium, hypertension, immunohistochemistry, spontaneously hypertensive rats (SHR).

Introduction

Hypertension is a common condition with important social and clinical significance, which is characterized by various morphological alterations in the target organs of the cardiovascular and urinary system, associated with a decrease in the functional capacity of the heart and kidney. These changes include left ventricular hypertrophy, cardiac fibrosis, pronounced glomerulosclerosis, thickening of the glomerular and tubular basement membranes, as well as renal interstitial fibrosis (1–3). Fibrosis is mainly of the reactive type, but a co-existence between reactive and reparative cannot be excluded (4).

Renal alterations described above are nonspecific and can be observed under different pathological conditions (5, 6). The re-

nal interstitium is a dynamic and heterogeneous structure, which contains various molecules such as collagen, glycosaminoglycans and glycoproteins. Collagen types I, III, V, VI, VII and XV are all expressed in the kidney under physiological conditions. Collagen type I is a typical interstitial protein (7). Collagen type III is normally poorly represented in the interstitium and is virtually not expressed in the glomeruli (8). The immunohistochemical distribution of collagen type V shows that it can be interspersed between other extracellular molecules or as separate fibers in the renal interstitium (7). The development of renal interstitial fibrosis depends on the balance between collagen synthesis and degradation. The expansion of extracellular molecules is a multifactorial process, in which various cell types, altered activity of matrix metalloproteinases, oxidative stress and profibrotic cytokines play a primary role. The renal interstitial fibroblasts are chiefly responsible for collagen synthesis and as such participate in the formation of the fibrous skeleton of the kidney (9, 10–13). Under pathological conditions, these cells may change into cells of the myofibroblastic phenotype and may be associated with the production of collagen type III (14). There exists an evidence that myofibroblasts play a crucial role in the development of interstitial fibrosis, which is observed in hypertensive kidney damage (15, 16).

The most abundant collagen fibrils in the extracellular matrix (ECM) of the myocardium are made up of collagen type I (4). They represent approximately 80 to 85 % of all cardiac collagen and provide strength and endurance to the heart wall. The second

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most abundant collagen type is type III, which represents around 11 to 12 % of all collagen fibrils. Its main function is to provide resilience and elasticity to the heart wall (17, 18). There is insufficient information about collagen type V in the literature, however its normal values are below 5 % of the total collagen mass in the cardiac wall and it is associated with collagen type I (19, 20). Heart wall hypertrophy occurs primarily due to cardiomyocytes growth in the conditions of high blood pressure. However, an excessive accumulation of fibrous tissue, mainly collagen fibrils, is also critical for the development of hypertrophy (19, 21, 22). Cardiac fibrosis is also characterized by qualitative changes in the collagen fibrils, especially in the collagen type I/III ratio (4, 17, 23, 24). Due to those changes in the composition of the collagen fibrils, the rigidity of the heart wall increases as cardiac fibrosis develops (25).

A review of the pertinent literature shows that data on the changes in the synthesis of the various collagen types in the target organs of hypertensive damage, in particular the kidney and heart, are relatively scarce. The spontaneously hypertensive rat (SHR) is a good experimental model for demonstration of the target organ damage of prolonged and untreated essential hypertension (26). Therefore, the aim of the present study was to investigate the immunohistochemical expression of selected collagen types, namely collagen types I and V and procollagen type III in the renal parenchyma and interstitium and in the myocardium of 6- and 12-month-old SHR.

Material and methods

For the present study, we used two age groups of 6- and 12-month-old SHR. These age periods correspond to the periods of initial phase (6-month-old SHR) and chronic phase (12-month-old SHR) of cardiac hypertrophy and hypertensive kidney damage (23, 27). Each group consisted of three male rats randomly selected from a large population of SHR available at the Laboratory of the Department of Anatomy, Histology and Embryology. All animal procedures were in compliance with the guidelines of Directive 2010/63/EU of the European Parliament. All experiments were conducted with the approval of the University Committee on Animal Resources (No. 4866). All animals received humane care in compliance with the ‘Principles of laboratory animal care’ formu-

lated by the National Society for Medical Research and the ‘Guide for the care and use of laboratory animals’ prepared by the National Institute of Health (NIH publication No. 86–23, revised 1996).

The rats were anesthetized intraperitoneally with Thiopental 40 mg/kg b.w. The chest cavity was opened and transcardiac perfusion was done with 4 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. The hearts and kidneys were rapidly removed and were rinsed in oxygenated physiological saline for a few minutes. Next, they were fixed in a 10 % neutral phosphate buffered formalin solution for at least 24 h. The samples from the kidneys and ventricles were then dehydrated in increasing concentrations of alcohol (70 %, 80 %, 95 %, 100 %), cleared in xylene and embedded in paraffin. Sections were then cut on a microtome at a thickness of 5 µm. The immunohistochemical analysis of the expression of collagen types I and V and procollagen type III was done in accordance with standardized methods (28). For semi-quantitative analysis of the expression of collagen types I and V and procollagen type III, we followed the well-established protocol (3).

Results

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

In 6-month-old SHR the immunohistochemical expression of collagen type I was observed in the parietal layer of Bowman’s capsule of the renal corpuscles, as well as among the proximal and distal tubular segments (Fig. 1a). Immunostaining for procollagen type III was reported mainly in the tubulointerstitium, while the reaction appeared nearly absent in the structural elements of the renal corpuscles (Fig. 1b). The immunohistochemical reaction for collagen type V was observed in the glomeruli of the cortical, midcortical and juxtamedullary nephrons and the proximal and distal tubular segments of the renal cortex (Fig. 1c).

In 12-month-old SHR, collagen type I was expressed in the glomeruli and the Bowman’s capsule, as well as around small blood vessels and proximal and distal tubular segments (Fig. 1d). The immunoreactivity for procollagen type III appeared especially prominent in the renal corpuscles and proximal and distal tubules (Fig. 1e). The reaction for collagen type V was observed in the glomeruli and the tubular segments (Fig. 1f).

Tab. 1. Semi-quantitative analysis of the immunohistochemical expression of collagen type I and V and procollagen type III in kidney and myocardium of SHR. The percentage for each score represents the percentage of visual fields that the IHC profiler assigned this score to. SHR – spontaneously hypertensive rats.

Type of collagen/procollagen	Kidney		Myocardium	
	6-month-old SHR	12-month-old SHR	6-month-old SHR	12-month-old SHR
Collagen type I	Positive (2+) (9 %)	Positive (2+) (66 %)	Positive (2+) (3 %)	Positive (2+) (66 %)
	Low-positive (1+) (78 %)	Low-positive (1+) (29 %)	Low-positive (1+) (85 %)	Low-positive (1+) (22 %)
	Negative (0) (13 %)	Negative (0) (5 %)	Negative (0) (12 %)	Negative (0) (12 %)
Procollagen type III	Low-positive (1+) (52 %)	High-positive (3+) (71 %)	Positive (2+) (4 %)	Positive (2+) (8 %)
	Negative (0) (48 %)	Positive (2+) (15 %)	Low-positive (1+) (77 %)	Low-positive (1+) (81 %)
		Low-positive (1+) (14 %)	Negative (0) (19 %)	Negative (0) (11 %)
Collagen type V	Low-positive (1+) (42 %)	High-positive (3+) (64 %)	Low-positive (1+) (46 %)	Positive (2+) (47 %)
	Negative (0) (58 %)	Positive (2+) (23 %)	Negative (0) (54 %)	Low-positive (1+) (42 %)
		Low-positive (1+) (13 %)		Negative (0) (11 %)

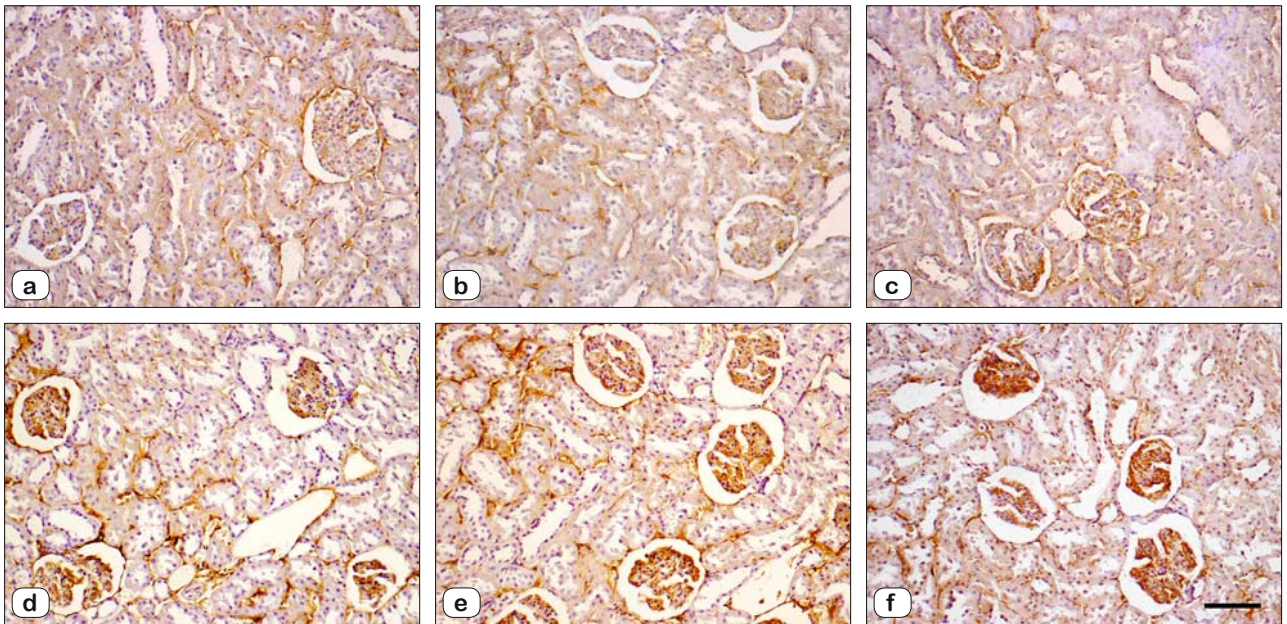


Fig. 1. Immunohistochemical expression of collagen type I and V and procollagen type III in the kidney of SHR. Scale bar – 100 μ m. a) Immunohistochemical expression of collagen type I in 6-month-old SHR. b) Immunohistochemical expression of procollagen type III in 6-month-old SHR. c) Immunohistochemical expression of collagen type V in 6-month-old SHR. d) Immunohistochemical expression of collagen type I in 12-month-old SHR. e) Immunohistochemical expression of procollagen type III in 12-month-old SHR. f) Immunohistochemical expression of collagen type V in 12-month-old SHR.

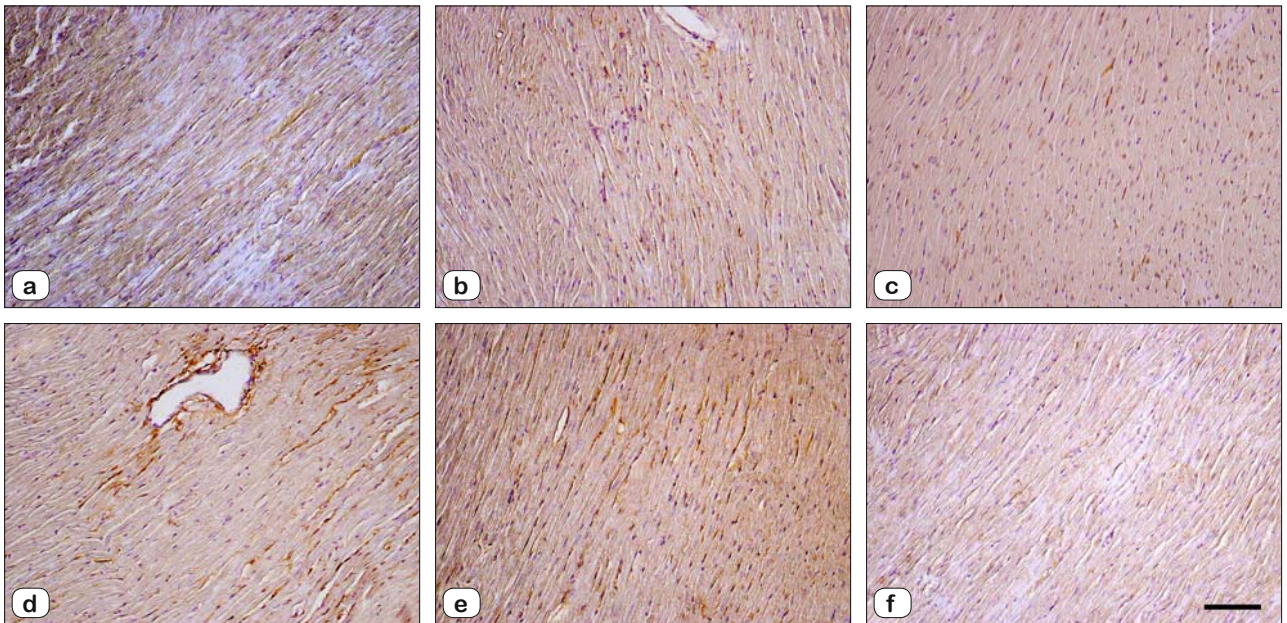


Fig. 2. Immunohistochemical expression of collagen type I and V and procollagen type III in the myocardium of SHR. Scale bar – 100 μ m. a) Immunohistochemical expression of collagen type I in 6-month-old SHR. b) Immunohistochemical expression of procollagen type III in 6-month-old SHR. c) Immunohistochemical expression of collagen type V in 6-month-old SHR. d) Immunohistochemical expression of collagen type I in 12-month-old SHR. e) Immunohistochemical expression of procollagen type III in 12-month-old SHR. f) Immunohistochemical expression of collagen type V in 12-month-old SHR.

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

The immunohistochemical staining for collagen type I showed a homogeneous pattern of distribution in 6-month-old SHR. It was

organized in the shape of individual coiled filamentous structures distributed between adjacent cardiomyocytes (Fig. 2a). Similar expression was reported for procollagen type III and collagen type V, but the reaction appeared less strong than that for type I.

Furthermore, we noted the presence of small accumulations of procollagen type III and collagen type V in the interstitium of the myocardium of the left ventricle (Figs 2b and 2c).

In 12-month-old SHR, immunoreactivity for collagen type I appeared higher compared to 6-month-old SHR and distribution of the collagen fibers was reported among the cardiomyocytes and around the blood vessels. We also found small deposits of type I in the interstitium (Fig. 2d). The alignment of the structural elements of procollagen type III in 12-month-old animals was similar to collagen type I, but the intensity of the reaction seemed lower (Fig. 2e). Immunostaining for collagen type V in 12-month-old SHR was characterized by single spiral-shaped fibers between the cardiac muscle cells (Fig. 2f).

Semi-quantitative analysis

As noted above, the intensity of the immunohistochemical reaction varied between the kidney and the myocardium 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.

Discussion

The present study reports the first data on the differential expression of collagen molecules in the kidney and heart as two main target organs of damage during the chronic phase of hypertension.

Our results showed that hypertensive kidney damage is characterized by pronounced accumulation of ECM molecules, especially procollagen types III and V. It has previously been suggested that upregulation of collagen types I and III is found in the initial process of renal fibrosis (29). Lu et al. (30) examined the morphological alterations in the glomerular capillary tufts and evaluated interstitial fibrosis in SHR compared to normotensive controls. They reported increased expression of collagen type III in 24-week-old SHR compared with 12-week-old SHR and observed almost no expression in normotensive animals. The immunohistochemical reaction was observed in the tubular epithelial cells, mesangial cells and interstitium (30). Our findings show a similar tendency for increased expression of procollagen type III as hypertension progresses. While in 6-month-old SHR the expression was mostly low-positive, in 12-month-old animals it was already predominantly high-positive. In addition, we have also demonstrated pronounced expression among the collecting ducts, especially in the region of the inner medulla.

Renal fibrosis is also characterized by increased expression of collagen type I, while in healthy rats, focal expression of collagen type I is found in the interstitium and around small blood vessels (31). In an experimental model of unilateral ureteral obstruction, the glomeruli show no positive reaction for collagen type I (31). These findings suggest that different collagen molecules are up-regulated depending on the etiology behind renal fibrosis. In SHR, we have demonstrated a positive reaction for collagen type I in the glomeruli and along the tubulointerstitium. It is well known that normally, the intraglomerular mesangial cells, as well as the cells of the glomerular filtration barrier cannot synthesize collagen type

I (32). On the contrary, glomerular injury is characterized precisely by deposition of collagen type I, which has been shown in the present study in case of hypertensive glomerulosclerosis. Liu et al. reported that the development of renal interstitial fibrosis in SHR is associated with increased expression of collagen types I, III and IV (33). The described changes in the expression of collagen types I and III are confirmed by the present study; however, we have also shown strong immunoreactivity for collagen type V in the older group of SHR, which indicates that this type also plays a role in hypertension-induced renal fibrosis. Indeed, there is limited information concerning the expression and distribution of collagen type V in the kidney under normal and pathological conditions. It is found mainly in the interstitium, while the mesangium and glomerular and tubular basement membranes show no positive reaction (34). Mesangial expression of collagen types III and V has been described in membranous nephropathy, Alport's syndrome and proliferative glomerulonephritis (35). Our study established that the renal cortex shows increased immunoreactivity for collagen type V in the glomerular capillary tufts and among proximal and distal tubular segments in older, 12-month-old SHR. In younger, 6-month-old SHR, collagen type V was negative to low-positive, which suggests that its deposition occurs in the late stages of hypertension. We should also note that the glomeruli with high-positive reaction exhibited the characteristic features of glomerulosclerosis—wrinkled glomerular capillary tufts and increased Bowman's capsule space.

One study in patients with chronic renal disease reported the distribution of various collagen types (36). Collagen types I and III were found mainly in the interstitium in controls, while higher expression of collagen types III and V was observed in patients. In addition, the authors suggested that the distribution of collagen type V correlates better with renal morphological alterations (36). Diabetic nephropathy is also associated with pronounced morphological changes in the renal corpuscles, which leads to glomerulosclerosis. An immunohistochemical study of the expression of collagen molecules in the glomeruli in patients with type 2 diabetes found that in the late stages of glomerulosclerosis there is an increased expression of collagen types III and V (37). The authors concluded that intraglomerular mesangial cells may play a crucial role in the development of diabetic glomerulosclerosis, because of phenotypic changes in these cells, which are characterized by increased production of collagen types III, V and VI. The distribution of various collagen molecules in the glomeruli shows that collagen type V is found in small amounts in the normal mesangium. In diabetic glomerulosclerosis, collagen types I and III are represented only in late glomerulosclerosis, coupled with an increased expression of type V (38). It is well known that the glomerular basement membrane is composed mainly of collagen type IV, but expression of type V has also been reported (39). Based on the results of the present study, hypertensive glomerulosclerosis is associated with an upregulation of procollagen type III, and collagen type III, respectively, which is normally not represented in the glomeruli, together with a high expression of collagen type V. The expression of these two collagen molecules shows similar changes in other pathological conditions, which lead to glomerulosclerosis.

Multiple studies have suggested that a key reason for myocardial hypertrophy is the remodeling of the ECM, mainly due to changes in collagen expression under hypertensive conditions (17, 19, 21–24). Our results confirmed this observation, as we reported changes in collagen expression in 6- and 12-month-old SHR. We found that the immunohistochemical expression of collagen type I in the ECM of the myocardium of 6-month-old animals was comparable to the normal values of distribution for that collagen type, corresponding to the data of other authors (23). Our semi-quantitative analysis in 12-month-old SHR showed higher expression of collagen type I compared to 6-month-old SHR in line with previous reports in the literature (4, 23, 25). With regard to type III, our results showed inconspicuous changes in the expression of procollagen type III over the 6 months difference in age of the two groups of SHR. This was consistent with the earlier study of Yang et al (23) who reported no alterations in collagen type III deposition throughout the chronic phase of hypertension. In contrast, Weber et al (19) observed significantly reduced concentration of collagen type I and much higher for collagen type III in 4-month-old SHR. These data significantly differ from our results and the one presented by Yang et al (23) but an obvious explanation could be the difference in age between the animals. At 4 months, hypertension is still developing and we would expect the structure of the myocardium to be quite well preserved, whereas at a later time, myocardial remodeling would lead to higher levels of collagen type I and increased rigidity of the heart wall. The last collagen type studied herein was collagen type V. We did not find previous studies in the literature on the expression of this specific collagen type in the myocardium under hypertensive conditions and have therefore presented the first data on its immunohistochemical expression and localization in the remodeled myocardium. We reported an increase in the expression of this type, probably due to its binding to collagen type I.

According to some authors, the most accurate indicator for myocardial hypertrophy and fibrosis is the collagen type I/III ratio (4, 17). Our findings suggest that the values of this ratio gradually go up due to the increased expression of collagen type I, while procollagen type III remained almost unaltered. Conversely, other studies have suggested that the ratio goes down, due to an upregulation of collagen type III and have stated that this is the hallmark for transition to heart failure (17, 19). An explanation for this discrepancy could be that our study focused on experimental models during the period of mature myocardial hypertrophy (6–12 months) (40, 41). In this period, we demonstrated that the expression of collagen type I was increased, which correlates with myocardial wall stiffness and rigidity (42). However, heart failure in SHR only develops after 18 months of age (43, 44). Therefore, upregulation of collagen type III and a shift in the type I/III ratio is only to be expected around that age period.

Limitations of the current study exist and should be noted. First, with regard to the myocardium, we only studied samples obtained from the left ventricle, whereas recent studies have pointed out to the changes in the right ventricle and the impact of systemic hypertension on right ventricle remodeling (3, 45–48). Second, the visual quantification of immunohistochemical images can be associated with significant inter-observer variation. In order

to resolve this issue, we used the IHC Profiler plugin for ImageJ software, which eliminates inter-observer visual perception bias. This method achieves 88.6 % correlation of scoring with blinded manual scoring as reported by trained pathologists ($p < 0.0001$, $CI = 95\%$) (28, 29). Nevertheless, it only allows for semi-quantitative assessment of immunostaining intensity. Last but not least, for the present study we only used male Wistar rats in an attempt to avoid the impact of cyclical changes in the female organism. However, the protective effect on the heart and kidney of estrogen hormones is well known and could possibly also affect myocardial remodeling and the expression of ECM molecules.

Conclusion

In conclusion, we have presented a comprehensive study on the expression of two types of collagen molecules and one procollagen molecule in the kidney and heart as target organs of damage under the conditions of systemic hypertension in SHR models. Our findings show a marked increase in the immunohistochemical expression of procollagen type III and collagen type V in the kidney of 12-month-old SHR as opposed to 6-month-old ones, thereby demonstrating the role played by these molecules in hypertensive kidney disease. In the myocardium, collagen types I and V were upregulated, while procollagen type III levels remained roughly the same, thus providing the molecular basis for increased heart wall rigidity and impaired myocardial relaxation.

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