Virchows Arch (1998) 432:267–277 

ORIGINAL ARTICLE

**Yichun Xu · Marie-Dominique Appay**
**Didier Heudes Robert Lemoine · Nicole Hinglais**
**Jean-Baptiste Michel · Jean Bariéty**

# Colocalization of collagen overexpression and inflammatory cell infiltration in the two-kidney one-clip rat model from the early days of hypertension onward

Received: 24 February 1997 / Accepted: 11 October 1997

**Abstract** In the first 6 days of hypertension, infiltrated mononuclear cells were colocalized with collagen (I) mRNA-overexpressing fibroblasts in the adventitial area of unclipped kidney. The number of adventitial infiltrated mononuclear cells was correlated with adventitial collagen (I) surface expansion. After 22 days of hypertension no collagen (I) mRNA-overexpressing fibroblasts or any increase in collagen area or mononuclear cell infiltration was observed. In the interstitium of unclipped kidney, collagen (I) mRNA overexpression, collagen (I) expansion and mononuclear cell infiltration began later, from the 7th day of hypertension, and kept increasing. In the clipped kidney, after expansion in the first 6 days of hypertension, the adventitial collagen remained stable. These results suggest that in the unclipped kidney fibroblastic activation begins within the first 6 days of hypertension in the adventitial area, but is transient, and fibrosis then spreads in the intersitium. Mononuclear cell infiltration is colocalized and correlated with adventitial and interstitial fibrosis. In the first 6 days, hypertension is not the only cause of fibrosis; the same level of adventitial fibrosis is detected in the nonhypertensive clipped kidney. All observed pathological phenomena could be detected within the first 3 days of hypertension

**Key words** Experimental hypertensive kidney · Adventitial, tubulo-interstitial fibrosis

## Introduction

Hypertensive nephrosclerosis (HNS) is one of the major causes of end-stage renal failure [38]. Glomerular and tubular lesions, vascular and interstitial fibrosis, and interstitial infiltration by mononuclear cells have all been well documented in HNS [9, 14, 24, 27, 41]. Nevertheless, the early cellular and fibrotic changes leading to HNS are still poorly understood. We have used the two-kidney one-clip (2K1C) as a model of HNS [41] to evaluate several phenomena appearing in the cortex during the first 31 days of high blood pressure (HBP). Collagen expansion around interlobular arteries and arterioles in the clipped and unclipped kidneys was quantified by morphometric analysis. Interstitial fibrosis, and mononuclear cell infiltration around arteries and arterioles in the unclipped kidney were quantitatively or semiquantitatively assessed. Cells synthesizing mRNAs for α1 collagen (I) and (IV) were located in the unclipped kidney by in situ hybridization.

Y. Xu · M.-D. Appay (✉) · D. Heudes · N. Hinglais · J. Bariéty
INSERM Unité 430, Hôpital Broussais, 96 rue Didot,
F-75 674 Paris Cedex 14, France,
Tel.: (+33) 01 43 95 95 95, ext. 3424, Fax: (+33) 01 45 45 90 59

R. Lemoine
Institut de Pathologie, CMU, Geneva 4, Switzerland

J.-B. Michel
INSERM Unité 460, UFR de Médecine X, Hôpital Bichat,
Paris Cedex 18, France

## Methods

Forty-seven male Wistar rats (Iffa-Credo, Lyon, France), 6 weeks old and weighing 140 g were used. All rats were anaesthetized with ether. A 0.2-mm clip was placed on the left renal artery in 29 rats and the right kidney was left untouched. The 18 control rats underwent sham surgery, including renal artery denudation without renal nerve cutting.

Animals were allowed free access to a regular pellet diet and water. Rats were weighed and systolic blood pressure measured by the tail cuff method (using a piezoelectric detection of caudal artery pulse downstream a cuff –W + W electronic recorder 8005, Apelab) twice weekly in awake rats. Five clipped rats were killed by decapitation on days 3, 7, 12, 17, 24, and 4 clipped rats, on day 38 after clipping. Three sham-operated rats were killed on each of the six dates. Most (20) of the 29 clipped rats were hypertensive when killed (systolic blood pressure >180 mmHg). Five clipped rats killed on day 3 after clipping and 4 rats killed on day 7 were not hypertensive and were excluded from the study. The systolic blood pressure of the sham-operated rats remained normal at 127 ± 10 mmHg (Fig. 1, top). As the effect of HBP on kidney structures was the critical parameter, all clipped rats were allocated to groups according to HBP length as follows. Group 1: 1–6 days of HBP ($n$ = 8), group 2: 7–17 days of HBP ($n$ = 8), group 3: 22–31 days of HBP ($n$ = 4)(Fig. 1, top). There were no groups with HBP lasting 17–21 days after clipping, since the onset of HBP is random.

Blood was collected at sacrifice and plasma renin activity (PRA) was measured by radioimmunoassay [26].

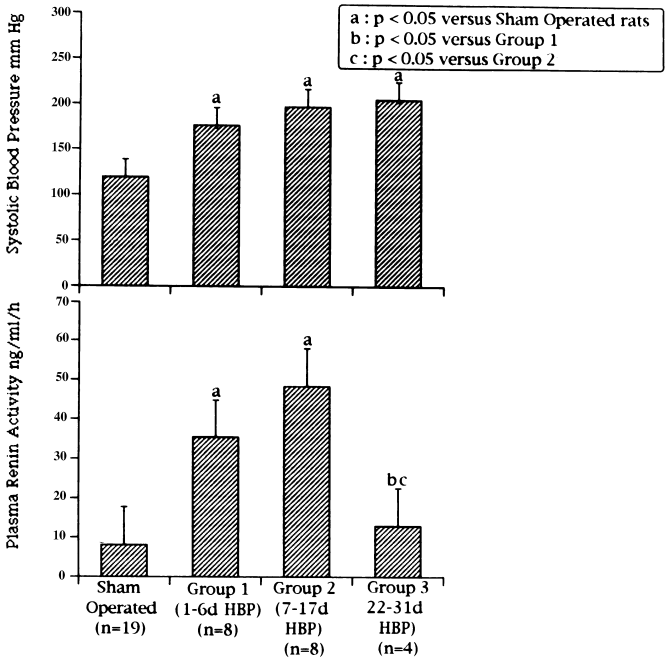



**Fig. 1** Changes in systolic blood pressure (*top*), and plasma renin activity (*bottom*) in sham-operated rats and in group 1 (1–6 days), group 2 (7–17 days) and group 3 (22–31 days) hypertensive rats in order of the duration of hypertension

The unclipped kidneys were divided into two parts. One part was frozen in liquid nitrogen and stored at –180°C for in situ hybridization and immunohistochemical studies, while the other was fixed in Dubosq–Brazil fixative for standard light microscopy and for collagen (I) quantitation using polarized light microscopy on picrosirius red stained sections. The clipped kidney was fixed in Dubosq–Brazil fixative for polarized light microscopy using picro sirius red staining.

Fixed tissue was dehydrated at room temperature through an ethanol series, embedded in paraffin and cut into 4-µm-thick sections. The sections from all rats were stained with haematoxylin and eosin, Masson's trichrome or picrosirius red. Picrosirius red has a high affinity for all types of collagen. Collagen (I) was identified using picrosirius red staining and polarized light microscopy. Polarizing microscopy of picrosirius red-stained sections reveals mostly collagen (I) as an orange-yellow colour with a 550-nm interference filter [19].

The fibrosis surrounding the interlobular arteries and cortical arterioles of clipped and unclipped kidneys was measured by quantitative morphometry of collagen (I) areas around arteries and arterioles on sections stained with picrosirius red. A video camera (Sony, Tokyo, Japan) was connected to an image analyser (Nachet 15000, Nachet, Evry, France) piloted by a microcomputer (Macintosh II, Apple, Cupertino, Calif.) running an image analysis program in C language. The investigator responsible for the morphometric analysis was unaware of the experimental group being analysed. An initial study of the running mean and of the running variance indicated that the measurement of 20 arteries (magnification ×250, calibration variables 0.52 µm/pixel) gave convergence to a good estimation of the quantitative parameters. Collagen (I) around arteries and arterioles connected to the media was measured for each field, separately from all distant interstitial collagen in the field. The area of collagen (I) and the perimeter of each artery were measured. The expansion of collagen (I) around arteries and arterioles was expressed as the ratio between the collagen area in square micrometres and the perimeter of the artery in micrometres (PAC/AP). This value was used to compare the fibrosis around arteries and arterioles independently of the section incidence.

Cortical interstitial fibrosis was evaluated semiquantitatively as the number of tubular sections surrounded by increased collagen (I) in the interstitium over 10 fields at a magnification of ×100 (3.04 $mm^2$ per field) for each rat in picrosirius red-stained sections in unclipped kidneys.

Polyclonal rabbit antibodies to mouse α1 collagen (IV) were obtained from Institut Pasteur (Lyon, France). Monoclonal antibody to rat α-smooth muscle cells (SMC) actin, a gift from Dr. Gabbiani (Faculty of Medicine, University of Geneva, Switzerland), were used to localize SMC and myofibroblasts [34]. The monoclonal antibody W3/25 (Crawley Down, Sussex, England) [40] and monoclonal antibody ED1 (Serotec, Oxford, England) [8] were used to identify identify T-helper lymphocytes and monocytes-macrophages, respectively.

Polyclonal rabbit antibodies to mouse α1 collagen (IV) were detected with goat anti-rabbit IgG coupled to fluorescein isothiocyanate (FITC; Institut Pasteur, Lyon, France). All primary monoclonal antibodies except the anti-rat α-SMC actin antibody were visualized with the alkaline phosphatase-anti-alkaline phosphatase (APAAP) complexes (Dakopatts, Copenhagen, Denmark) [5]. Endogenous alkaline phosphatase was inhibited by heating the slides to 80°C as reported elsewhere [12]. Anti-rat α-SMC actin monoclonal antibody was detected by the peroxidase anti-peroxidase (PAP) technique.

Cells labelled with ED1 and W3/25 surrounding 20 interlobular arterial or cortical arteriolar sections (×400) were counted to obtain the mean number of labelled cells per arterial section. The interstitial infiltrating cells (i.i.c.) were evaluated in interstitial areas using the following index: 0 no i.i.c.; 1 up to 50 i.i.c. dispersed in small clusters of 3–5 cells; 250–100 i.i.c. distributed into sheets; 3 over >100 i.i.c. uniformly distributed throughout the field. Ten fields (3,04 $µm^2$ at a magnification of ×100) were scanned per rat.

Two cDNA probes, one encoding the murine α1 collagen (I) chain (975 bp), and the other encoding the murine α1 collagen (IV) chain (700 bp) were provided by Dr. M.Y. Laurent (INSERM U 118, Paris, France) [11]. Cryostat sections (8 µm) were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 15 min, washed in PBS for 2 min and dehydrated in an ethanol series. The cDNA probes were labelled with $^{35}S$ dCTP using a random primer DNA labelling system (Gibco BRL, Cergy Pontoise, France) to a specific activity of $1.10^7$–$1.10^8$ cpm/µg. The hybridization mixture was: 50% formamide, 10 mM Tris, pH 7.4, 0.6 M NaCl, 1 mM EDTA, 1 × Denhardt solution, salmon sperm DNA (200 µg/ml), and herring sperm DNA (200 µg/ml), yeast t-RNA (500 µg/ml), 10% dextran sulfate, and 10 mM dithiothreitol. Hybridization mixture (15 µl containing 7.5 ng $^{35}S$-labelled denatured cDNA) was placed on each section. Sections were covered with siliconized glass coverslips, sealed, and incubated overnight at 41°C in a moist chamber. The tissue sections were then washed twice in 4 × saline sodium citrate (SSC) containing 40% formamide at 45°C for 15 min, then with 2 × SSC at 60°C for 30 min, and finally with 0.5 × SSC at room temperature for 1 h. The sections were dehydrated in an ethanol series, dipped in 50% $NTB_2$ emulsion (Eastman Kodak Company, Rochester, N.Y.) in water and exposed for 7–12 days in the dark at 4°C. The autoradiographs were developed in Kodak D 19, fixed in Kodak Rapid Fix and counterstained with haematoxylin and eosin. The kidneys of 4 clipped rats in group 1, 5 in group 2 and 4 in group 3 and of 5 sham-operated rats were used for in situ hybridization. Thirteen hypertensive rat kidneys and 5 sham-operated rat kidneys were hybridized in situ for α1 collagen (I and IV) mRNAs.

Control experiments included pretreatment of fixed sections with ribonuclease A (RNase A, 50 µg/ml) (Sigma, St Quentin Fallavier, France) in 2 × SSC for 30 min at 37°C prior to hybridization, and hybridization of slides with $^{35}S$-labelled pGEM plasmid.

All results are expressed as means ± SEM per group. All results were compared by one-way anova and confirmed by a non-parametric test (Kruskall–Wallis test or Mann–Whitney U-test). Correlations between quantitative parameters were assessed using the Spearman-test. Statistical significance was accepted at $P < 0.05$.

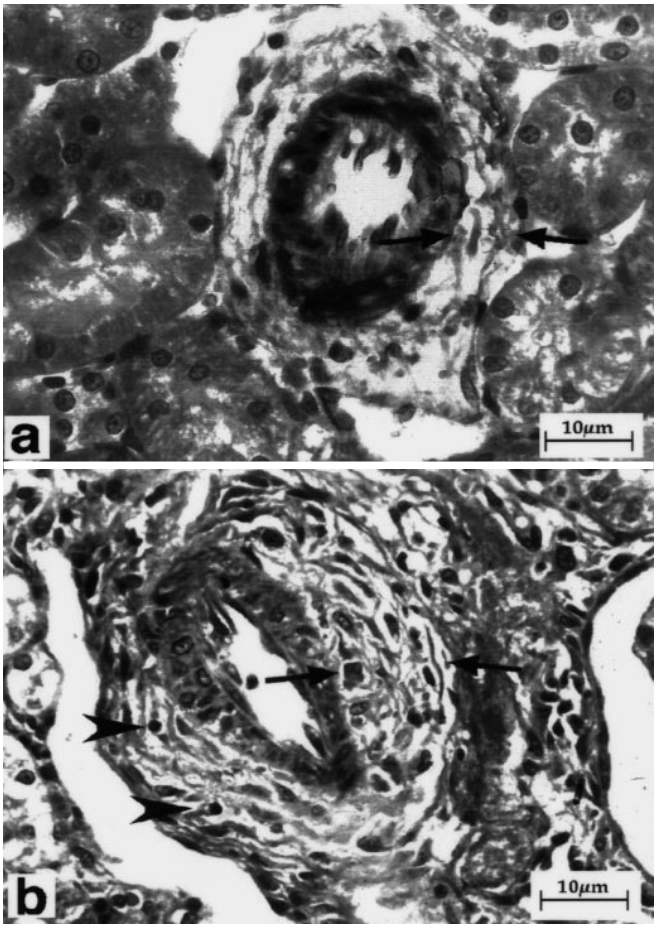



**Fig. 2** **a** Sham-operated rat. Interlobular artery. The adventitia is normal (between *arrows*). **b** Unclipped kidney of a rat killed after 7 days of hypertension (group 2). Interlobular artery. The adventitia is greatly enlarged by proliferative fibroblasts, separated by layers of extracellular matrix (between *arrows*). The enlarged adventitia is infiltrated by mononuclear cells (*arrowheads*). There is no medial or intimal smooth muscle cell proliferation. Light microscopy, Masson's trichrome

## Results

The PRA was normal at 11.9 ± 1.57 ng angiotensin I/ml per hour in sham-operated control rats. The PRA was significantly more elevated (35.53 ± 6.83 ng angiotensin I/ml per hour) in group 1 than in sham-operated rats, and reached 46.66 ± 11.24 ng angiotensin I/ml per h in group 2. It decreased thereafter in group 3 (18.4 ± 4.49 ng angiotensin I/ml per h as seen in Fig. 1 (bottom).

In the 18 sham-operated rats, the renal tissue was normal. Renal lesions were observed in clipped and unclipped kidneys of hypertensive rats.

Glomerular lesions were moderate and focal and occurred only in groups 2 and 3. The most frequent abnormality was the presence of podocytic hyalin droplets. Ischaemic and necrotic glomeruli were rare.

Necroses of the media of some interlobular arteries and cortical arterioles were detected in the unclipped kidneys of the three groups of rats.

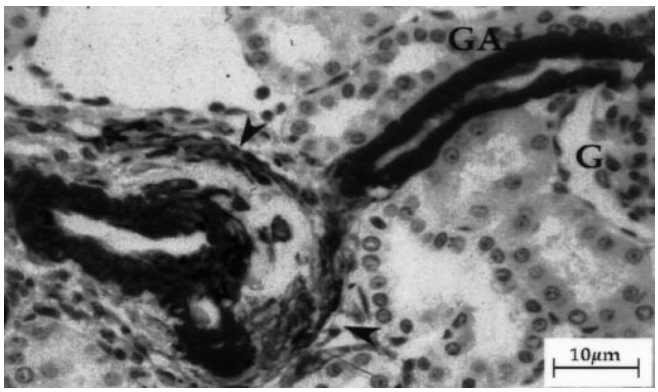



**Fig. 3** Immunohistochemical detection of smooth muscle cells (SMC) with anti-α-SMC actin monoclonal antibody (peroxidase-anti-peroxidase technique) on the unclipped kidney of an hypertensive rat killed 14 days after hypertension onset (group 2). The α-SMC actin-positive smooth muscle cells of the arterial and juxtaglomerular arteriolar (*GA*) media are neither hypertrophic nor hyperplasic. In the interlobular artery (*A*), proliferating fibroblasts are α-SMC actin-positive, especially at the periphery of the onion-skin-like lesion (*arrowheads*). *G* glomerulus

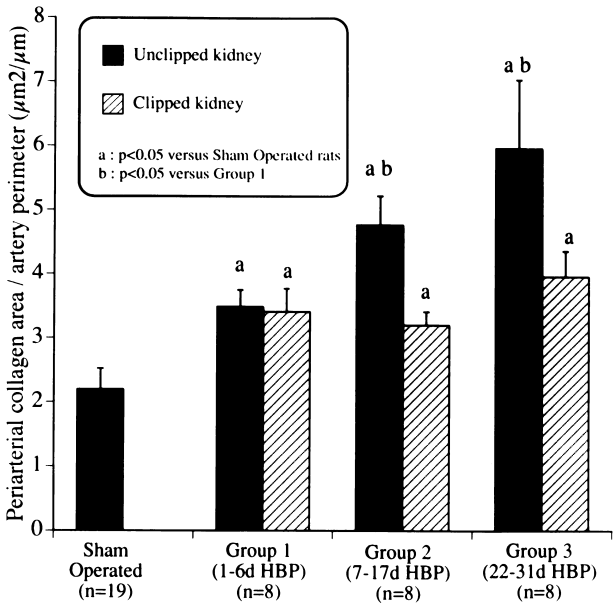



**Fig. 4** Morphometric measurement of periarterial collagen I area on kidney sections stained with picrosirius red, and examined with polarized light microscopy. The periarterial collagen I area in the unclipped kidneys increases with increasing duration of hypertension. The periarterial collagen I area remains significantly higher in the clipped kidneys than in the sham-operated rats, but does not increase with the duration of hypertension

Interlobular arteries and glomerular arterioles were surrounded by round mononuclear cells as early as the 3rd day of HBP (group 1). In groups 2 and 3, the numerous proliferating fibroblasts and round mononuclear cells were separated by concentric layers of extracellular matrix, mimicking onion-skin-like lesions (Fig. 2). Some of these proliferating fibroblasts were positive with anti-α-SMC antibody, particularly at the periphery of the onion-

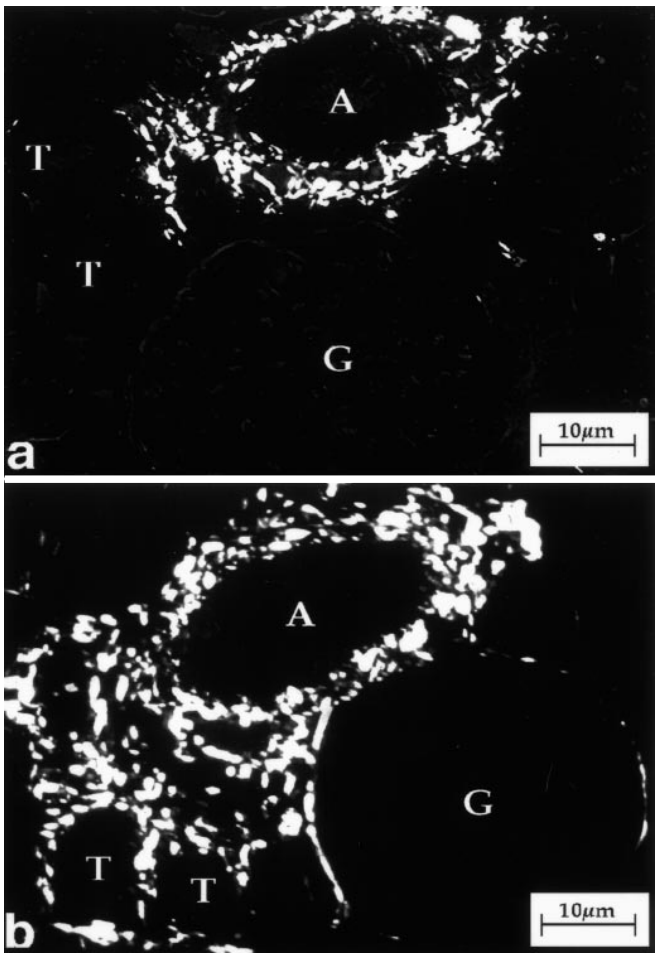



**Fig. 5** **a** Polarized light microscopy using a 550 nm filter on picrosirius red-stained kidney sections to detect collagen I. **a** Sham-operated rat. Adventitial collagen I around an artery (*A*) does not extend into the interstitium and around tubules (*T*) and glomerulus (*G*). **b** Unclipped kidney of rat killed after 14 days of hypertension (group 2). Some adventitial collagen I around an artery (*A*) extends into the interstitium and around tubules (*T*) and glomerulus (*G*)

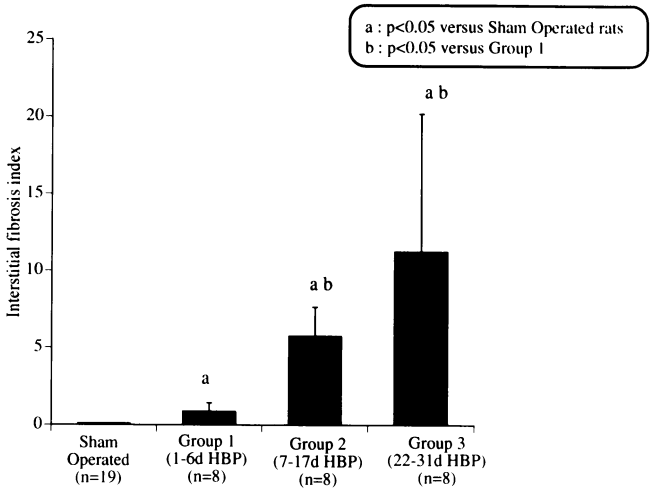



**Fig. 6** Interstitial fibrosis evaluated by semi-quantitation of interstitial collagen I in unclipped kidney. The kidney sections stained with picrosirius red are observed by polarized light microscopy. The interstitial fibrosis is significantly more marked in all the three groups of hypertensive rats than in sham-operated rats, and in groups 2 and 3 than in group 1

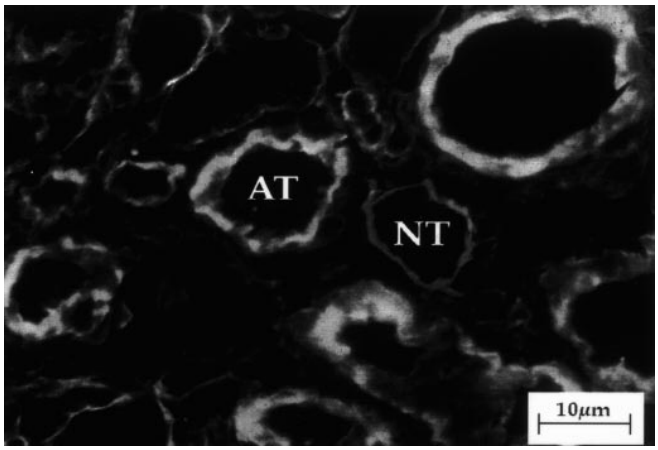



**Fig. 7** Immunofluorescence using antibodies to α1(IV) collagen. Unclipped kidney of rat killed after 28 days of hypertension (group 3). The thickened tubular basement membranes are labelled and have a wrinkled appearance (*AT* altered tubule) when compared to normal tubules (*NT*)

skin-like lesions (Fig. 3). The SMC in the media of these arterial sections were neither more hypertrophic nor more hyperplasic than in sham-operated rats (Figs. 2, 3).

The ratio between the adventitial area of collagen (I) and the arterial perimeter (PAC/AP) was significantly greater in group 1 ($3.48 \pm 0.26$ μm²/μm) than in sham-operated rats ($2.19 \pm 0.32$ μm²/μm). The PAC/AP value was significantly greater in group 2 than in group 1, but not in group 3 than in group 2 (Fig. 4). Adventitial collagen (I) gradually extended into the interstitium and around tubules and glomeruli in some areas (Fig. 5). Some arteries were surrounded by normal areas of collagen in all hypertensive groups. The PAC/AP in the clipped kidney was significantly greater in group 1 than in sham-operated rats, and did not increase with the duration of HBP (Fig. 4).

The collagen (I) in the interstitium increased significantly with HBP in groups 1, 2 and 3 compared to sham-operated rats (Fig. 6). Interstitial collagen (I) was significantly greater in groups 2 and 3 than in group 1. The altered tubules in these fibrotic areas had thickened basal laminae with enlarged lumina and small cuboid epithelial cells. These enlarged basal laminae stained strongly with antibody to α1 collagen (IV) (Fig. 7). There was no noticeable labelling with antibody to α1 collagen (IV) in the interstitium or in the periarterial/periarteriolar area (Fig. 7). Some fibroblasts around altered tubules were α-SMC actin positive (data not shown).

Some fibroblasts in adventitial monolayers in the periarterial/periarteriolar area of sham-operated rats ($n = 5$) expressed collagen (I) mRNAs (Fig. 8a, b). In group 1 rats ($n = 4$), the adventitial fibroblasts in multilayers gave intense signal around arteries and arterioles with

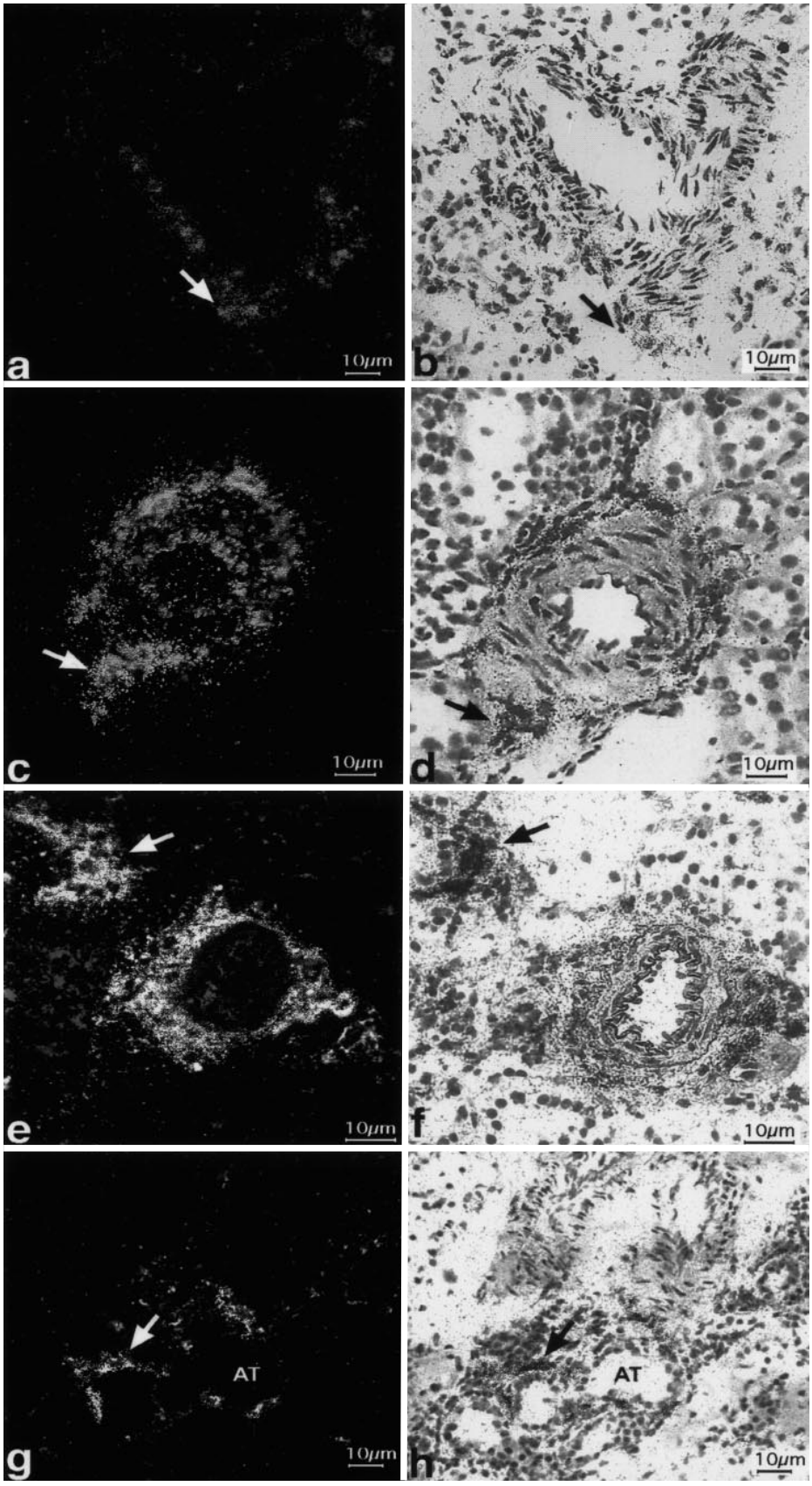
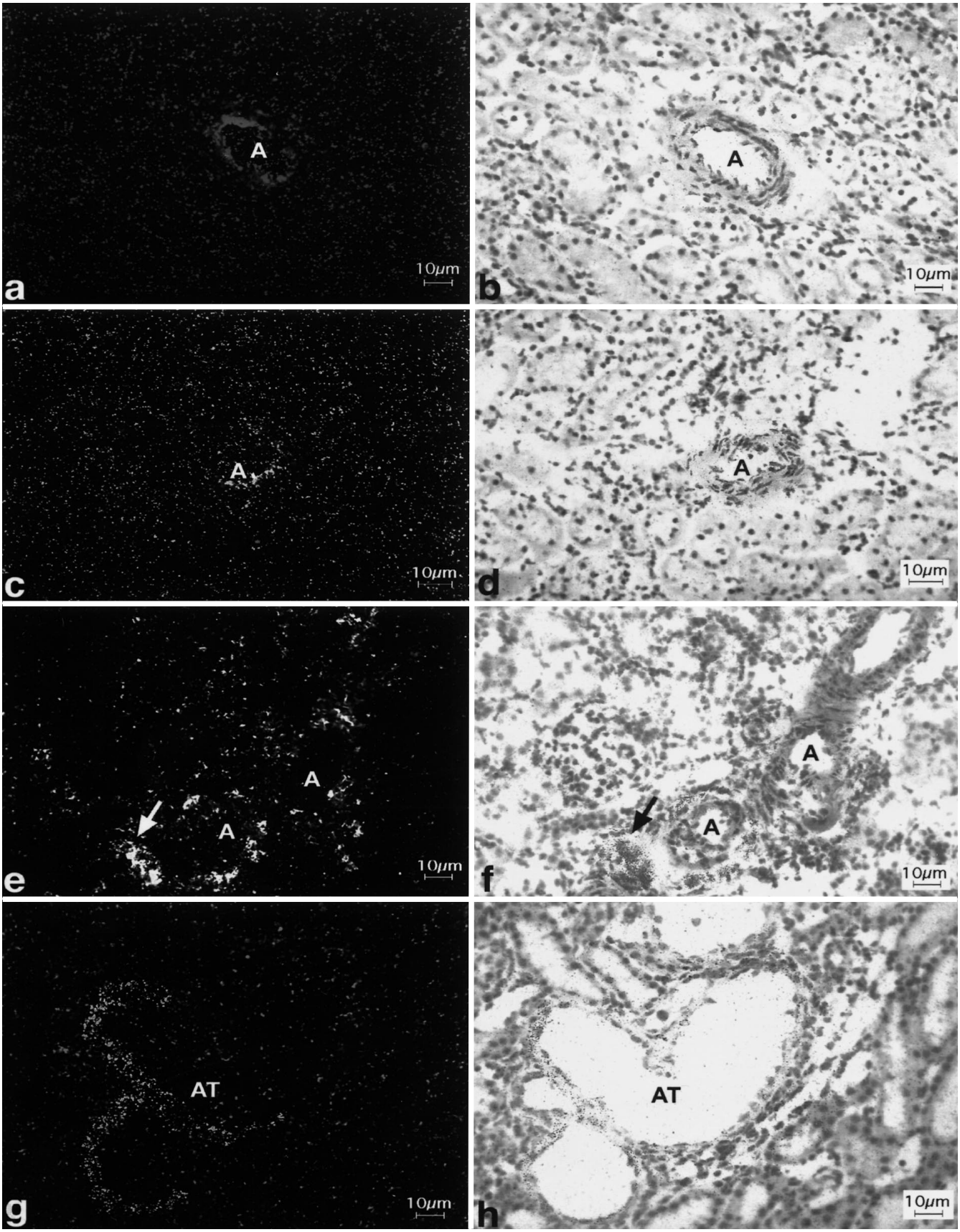



**Fig. 8a–h** In situ hybridization of α1(I) collagen mRNA with α1(I) collagen cDNA probe in unclipped kidneys. *Left* dark-field micrographs showing intensity of the hybridization signal; *right* paired bright-field micrographs showing anatomical localization of that signal. **a, b** In sham-operated rat, interlobular artery showing α1(I) collagen mRNAs in fibroblasts (*arrow*) in the adventitia. **c, d** In a hypertensive rat killed on the 1st day of hypertension (group 1), artery showing many α1(I) collagen mRNAs in fibroblasts in the adventitia (*arrow*). **e, f** In hypertensive rat killed on the 14th day of hypertension (group 2), interlobular artery showing many α1(I) collagen mRNAs in fibroblasts in the adventitia (*arrow*) and many intensely α1(I) collagen mRNA-containing fibroblasts (*arrowhead*) around tubules. **g, h** In hypertensive rat killed on the 28th day of hypertension (group 3), numerous α1(I) collagen mRNA-containing fibroblasts (*arrow*) detected in the interstitium and around altered tubules (*AT*)


a
A
10μm
b
A
10μm
c
A
10μm
d
A
10μm
e
A
A
10μm
f
A
A
10μm
g
AT
10μm
h
AT
10μm

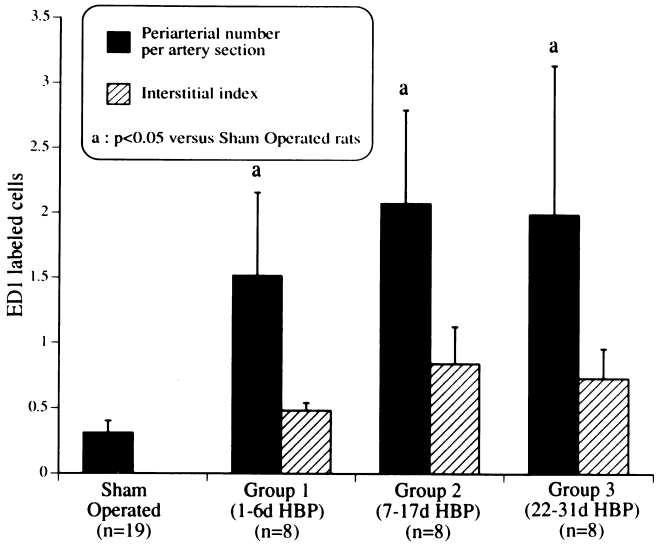



**Fig. 10** Immunohistochemical quantitation of periarterial/periarteriolar ED1-labelled cells and semiquantitative index of interstitial ED1-labelled cells from kidney of sham-operated rats and from unclipped kidney of rats in groups 1, 2, and 3. The number of periarterial/periarteriolar ED1-labelled cells is significantly higher in groups 1 and 2 than in sham-operated rats. The index of interstitial ED1-labelled cells was significantly higher in the three hypertensive groups than in sham-operated rats

normal (Fig. 8c, d) or necrotic media. In group 2 rats ($n = 5$), the adventitial signal was more intense (Fig. 8e, f) and there were also some clusters of α1 collagen (I) mRNA-expressing fibroblasts in the cortical interstitium around tubules in (Fig. 8e, f). In group 3 rats ($n = 4$), no α1 collagen (I) mRNA-expressing fibroblasts were observed around arteries and arterioles, but such fibroblasts were still present in the interstitium (Fig. 8g, h).

None of the structures in the kidneys of sham-operated (Fig. 9a, b) or group 1 (Fig. 9c, d) rats expressed α1 collagen (IV) mRNAs, but 4 of the 13 hypertensive rats had fibroblasts containing α1 collagen (IV) mRNAs in the adventitial periarterial/periarteriolar area. This α1 collagen (IV) mRNA expression was transient; it was detected on day 7 (group 2) of hypertension, maximal on day 14 (group 2; Fig. 9e, f) and absent on day 28 (group 3). Five hypertensive rats gave α1 collagen (IV) mRNA expression in the epithelial cells of altered tubules and in the interstitial fibroblasts in contact with them. These α1 collagen (IV) mRNA-expressing cells were present from day 7 (group 2) of hypertension onward, and were more numerous on day 28 (group 3) (Fig. 9 g, h).

◀ **Fig. 9a–h** In situ hybridization of α1(IV) collagen mRNA with α1(IV) collagen cDNA probe in unclipped kidneys. *Left* dark-field micrographs showing intensity of the hybridization signal; *right* paired bright-field micrographs showing anatomical localization of that signal. **a, b** No signal is detected in sham-operated rats or in **c, d** group 1 rats. **e, f** In rats killed after 7 days of hypertension (group 2), α1(IV) collagen mRNAs are detected in adventitial fibroblasts (*arrow*). **g, h** In rat killed after 28 days of hypertension (group 3). α1(IV) collagen mRNAs are detected in altered tubular epithelial cells. (*A* artery, *AT* altered tubules)

Sham-operated rats had dispersed cortical periarterial/periarteriolar and interstitial cells labelled with anti-ED1 or anti-W3/25 antibodies. W3/25-labelled cells were more numerous than ED1-labelled cells. There were significantly more periarterial/periarteriolar ED1-labelled cells in groups 1, and 2 than in sham-operated rats (Fig. 10). The mean number of ED1-labelled cells per arterial and arteriolar section was positively and significantly correlated ($r = 0.68$, $P < 0.01$) with PAC/AP (Fig. 11a). The semiquantitative measurement of interstitial ED1-labelled cells was significantly greater in the three hypertensive groups than in the sham-operated rats (Fig. 10). The highest index, 0.84 ± 0.18 labelled cell per interstitial field, was reached in group 2, but it was not significantly different from the values in groups 1 and 3 (Fig. 10). The semiquantitative assessment of interstitial ED1-labelled cells was also significantly correlated ($r = 0.75$, $P < 0.001$) with PAC/AP (Fig. 11b).

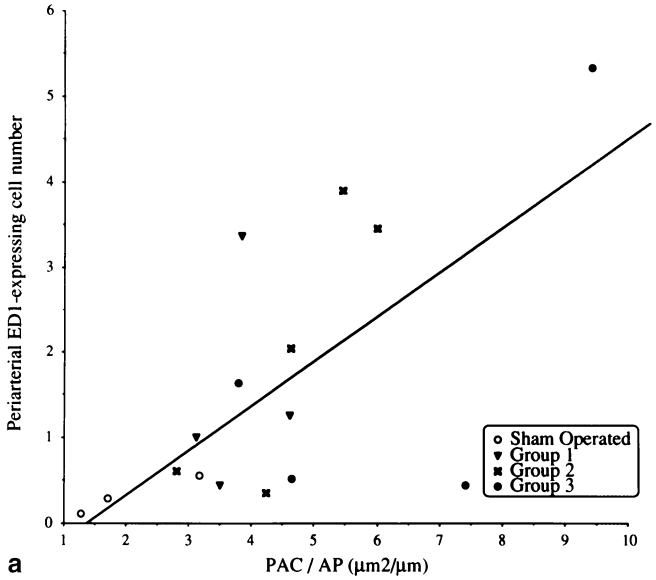
The highest value for periarterial/periarteriolar W3/25-expressing cells, 8.52 ± 6.26, was reached in group 2. It was significantly higher than that in sham-operated rats (Fig. 12). The mean number of periarterial/periarteriolar W3/25-labelled cells was not significantly correlated ($r = 0.39$) with PAC/AP. As for ED1-labelled cells, the number varied greatly from one vessel section to another, and could reach 50 cells per vessel section. Semiquantitative measurement of the mean number of interstitial W3/25-labelled cells showed significant increases in groups 2 (1.49 ± 0.02) and 3 (1.58 ± 0.12) over the number in the sham-operated group (Fig. 12). The index of W3/25-labelled cells per interstitial field was significantly correlated ($r = 0.72$, $P < 0.01$) with PAC/AP (Fig. 11c).

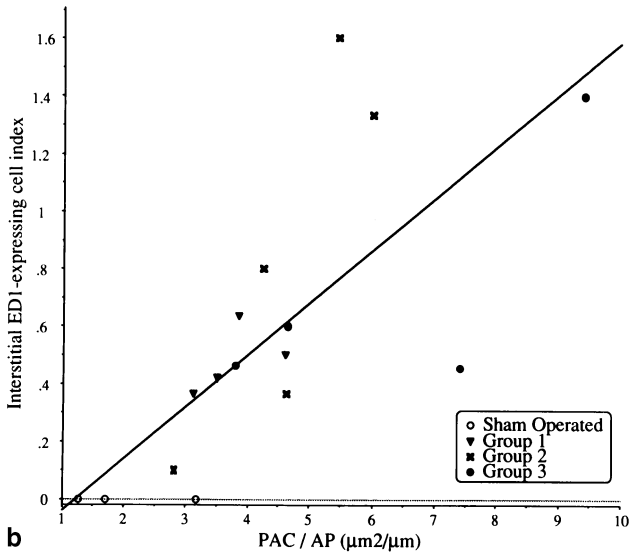
## Discussion

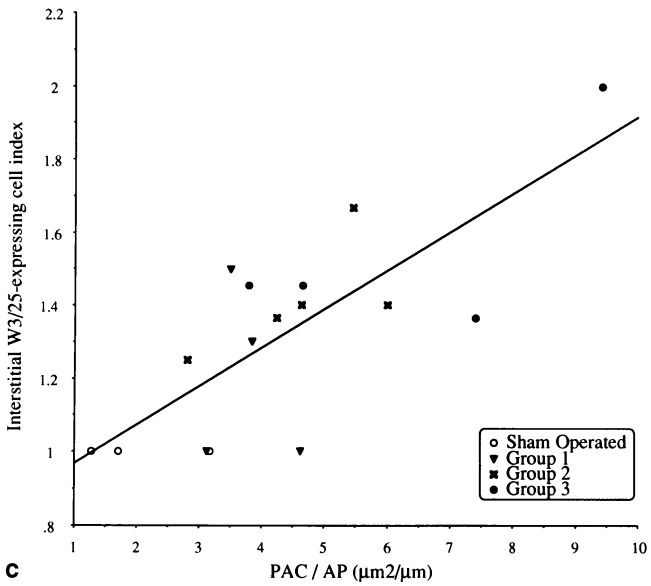
The unclipped kidney in the 2KC1C model of hypertensive rats is a well-documented model of HNS, in which there are arterial, glomerular, tubular, and interstitial lesions [9, 14, 24, 27, 41], but the early cellular and fibrotic events that initiate the HNS are poorly understood.

Within the first 6 days of HBP (group 1) the following changes occurred in the periarterial/periarteriolar area of the unclipped kidney: an increase in the area of collagen (I); an increase of collagen (I) mRNAs in proliferative adventitial fibroblasts; and an increase in ED1-expressing cells (monocytes/macrophages). Patchy medial necroses affect arteries and arterioles. All these modifications could be observed as early as the first 3 days of hypertension.

After 7–17 days of HBP (group 2), the unclipped kidney contained even more adventitial collagen (I), and collagen (I) mRNA expression was at its highest. The number of ED1- and W3/25-expressing cells increased around arteries. An increase of collagen (I) area occurred in the interstitium, paralleling an increase in fibroblasts expressing collagen (I and IV) mRNAs, and correlated with the number of ED1- and W3/25-expressing cells.



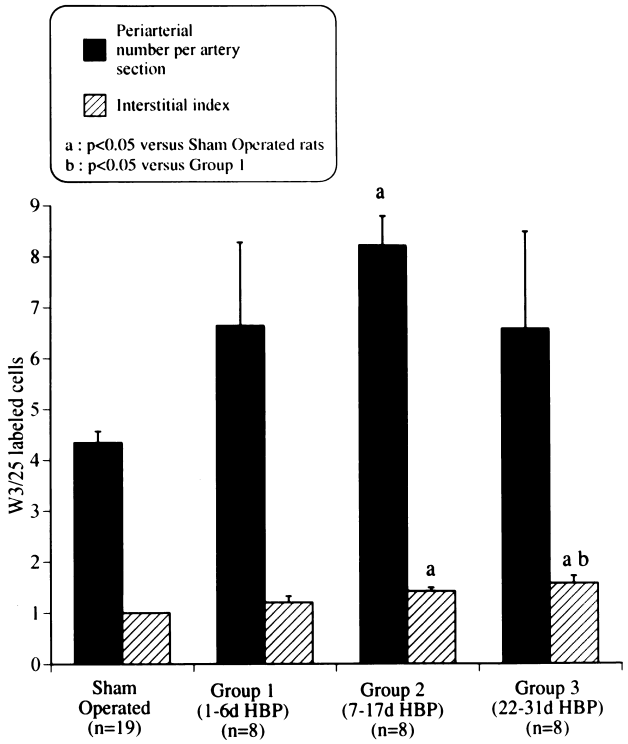




**Fig. 12** Immunohistochemical quantitation of periarterial/periarteriolar W3/25-labelled cells in kidney tissue sections and semi-quantitative index of interstitial W3/25-labelled cells from sham-operated rats and from unclipped kidney of rats in groups 1, 2 and 3. The number of periarterial W3/25-labelled cells is significantly higher in group 2 than in sham-operated rats. The index of interstitial W3/25-labelled cells was significantly higher in groups 2 and 3 than in sham-operated rats

After 22–31 days of HBP (group 3), in the periarterial/periarteriolar area of unclipped kidneys, collagen (I) area was increasing versus group 2. The numbers of ED1- and W3/25-expressing cells remained high and stable, and collagen (I) mRNAs were no longer detectable. In the interstitium, collagen (I and IV) mRNAs were always overexpressed. Collagen (I) area and ED1 and W3/25 cell numbers remained high.

In the clipped kidney, the area of periarterial/periarteriolar collagen (I) was stable in groups 1, 2 and 3, but significantly larger than in sham-operated rats.

Adventitial fibrosis without SMC proliferation in the media has also been observed by others in the unclipped kidney of 2K1C hypertension [24], and in extrarenal vessels [2, 20, 29]. Adventitial proliferative and fibrotic arterial lesions have also been described in the coronary lesions [15] and cerebral arterial lesions [11] of spontaneously hypertensive rats.

The very early periarterial/periarteriolar accumulation of extracellular matrix in the unclipped kidney has been

**Fig. 11** Correlations in the unclipped kidneys between PAC/AP and **a** the mean number of ED1-expressing cells around arteries and arterioles ($r = 0.67$, $P < 0.01$), **b** the index of interstitial ED1-expressing cells ($r = 0.72$, $P < 0.01$), and **c** the index of interstitial W3/25-expressing cells ($r = 0.70$ and $P < 0.01$)

assessed semiquantitavely in other studies [9, 17, 24, 41]. In the Helmchen study [14], medial thickness but not adventitial fibrosis was measured morphometrically in the days immediately after clipping. The authors considered this medial thickness to be due to intracellular oedema. Using computer-assisted morphometric analysis, we have measured adventitial collagen separately from interstitial fibrosis and have demonstrated that there is a significant increase in adventitial collagen (I) accompanied by collagen (I) mRNA overexpression in the first 6 days of hypertension. This induction of periarterial/periarteriolar fibrosis in the unclipped kidney is not only early but also transient; collagen (I) mRNAs were no longer detected after 17 days of HBP, and there was no further significant increase in fibrosis in our study.

In the 2K1C model of HNS, the effects of haemodynamic factors bound to HBP are difficult to dissociate from those of angiotensin II (Ang II). It would have been of interest to compare arterial and arteriolar lesions in the 2K1C model with high PRA with the same lesions in a model of hypertension with low PRA (Doca salt intoxication). Unfortunately, the comparison is difficult, since hypertension level and duration are not the same as ours in the available studies [33, 43]. Nevertheless, in these studies it seems that adventitial perivascular lesions are not predominant, suggesting that the renin-angiotensin system participates specifically in the development of the adventitial vascular lesions [43] .

The activation of the renin-angiotensin system (RAS) that occurs in our model, as indicated by PRA measurements, may be a triggering factor for fibrosis. Although the RAS is activated mainly in the clipped kidney, it has a potential effect on both kidneys [9, 13]. The fibrotic activity of systemic and local Ang II has been widely discussed. However, in vitro effects of Ang II on renal fibroblast activation are poorly documented and remain controversial. AT1 receptors for Ang II have been described in rat cultured cardiac fibroblasts [6, 25, 32, 37]. Adding Ang II to the culture medium causes both cardiac fibroblast proliferation [1, 31, 32] and collagen synthesis [4, 6, 44], but Ang II has no effect on the growth of murine fibroblasts isolated from the renal tubulo-interstitium [42]. In vivo inhibition of Ang II production by converting enzyme inhibitors (CEI) [27] or inhibition of Ang II binding by DuP 753 (losartan) [29] does not dissociate direct effects of Ang II from those of HBP, since the two phenomena are tightly linked. However, the adventitial fibrosis of the nonhypertensive clipped kidneys remained unchanged in all three hypertensive groups. Roughly similar observations were reported by Eng et al. after 11 weeks of clipping [9]. In our studies, fibrosis significantly increases in the hypertensive unclipped kidney from day 6 to day 17. Thus dissociation between hormonal effects of Ang II, which is elevated in both kidneys [9, 13], and haemodynamic effects of blood pressure, which is elevated in the unclipped kidney, indicates that blood pressure is a potent triggering factor for fibrosis. Nevertheless, the small but significant enlargement of the adventitial area of collagen (I) in the nonhypertensive clipped kidney in the first few days of HBP could be explained by the action of Ang II. In fact, several in vivo studies suggest that Ang II could mediate arterial SMC proliferation independently of HBP [17, 23, 30]. Moreover, in experimental renovascular hypertension Brilla et al. [3] noticed that myocardial fibrosis occurred in not only the pressure-overloaded, hypertrophied left ventricle, but also in the normotensive, nonhypertrophied right ventricle, suggesting that a circulating hormonal factor (Ang II), and not haemodynamic factors, is responsible for this adverse fibrous tissue response.

Inflammation could be another potent triggering factor for fibrosis, since suppression of macrophages leads to the suppression of fibrogenesis in several experimental models [7, 10, 18, 22, 36]. We also found a significant correlation between the number of ED1-expressing cells and the adventitial area of collagen (I). However, there is as yet no indication as to why these infiltrating cells are attracted into these areas around arteries and arterioles as early as the 6th day of HBP.

Fibrosis occurs later in the interstitium than in the adventitial area, that is on day 7 of HBP, and regularly increases with the duration of HBP. This interstitial fibrosis is accompanied by overexpression of collagen (I) mRNAs in interstitial fibroblasts. This interstitial fibrosis is a continuous phenomenon, unlike the transient periarterial/periarteriolar fibrosis. As with adventitial fibrosis, interstitial fibrosis is accompanied by an increase in interstitial infiltrating lymphocytes and macrophages in the same sites as $\alpha$1 collagen (I) and (IV) mRNA-expressing fibroblasts. The number of these infiltrating cells is also correlated with the extension of adventitial fibrosis. The phenotypic distribution of these infiltrating cells is in agreement with the data of Mai [24] in 2K1C hypertension, but also of other experimental [21, 35] and human sclerosing nephropathies [39]. The interstitial fibrosis seems to progress morphologically from the periarterial area towards the interstitium, much like the process reported by Wiggins et al. [16, 39] in experimental glomerulonephritis. The authors hypothetise that during this type of renal injury with an inflammatory process, (myo)fibroblasts could be stimulated to synthetise collagen and to migrate into the interstitial space, leading to fibrosis [39]. The fact that many fibroblasts express $\alpha$-SMC actin in interstitial and adventitial fibrosis suggests that these cells display a myofibroblastic differentiation. The reasons for such differentiation are not clear, but have been observed in different circumstances: Ang II infusion [17], obstructive nephropathy [28], and 2K1C model [9].

The overexpression of collagen (IV) mRNAs in tubular cells that begins on the 7th day of HBP is accompanied by overexpression of collagen (IV) in enlarged tubular basement membranes. However, the collagen (IV) mRNAs in interstitial fibroblasts are not accompanied by expression of collagen (IV) protein, or at least this is not detected with the antibody used. This apparent contradiction may be due to a postranscriptional blockade of pro-

tein synthesis or to a synthesis of collagen (IV) after more than 28 days of HBP. In fact, collagen (IV) was detected by Eng et al. [9] only 11 weeks after clipping.

In summary, morphometric analysis and in situ hybridization studies show that fibrosis begins early in this model of HNS, within the first 6 days of hypertension, and is restricted to the periarterial/periarteriolar areas of the unclipped kidney. The process extends to the interstitium after 7 days. HBP is involved in adventitial fibrosis after the 7th day. Nevertheless, in the first 6 days HBP is not the only relevant factor for fibrosis; the adventitial collagen expansion is identical in both clipped and unclipped kidneys. The colocalization of infiltrating cells and activated α1 collagen (I)-expressing fibroblasts and the correlation between the level of inflammatory cells and fibrosis suggest that inflammation is involved in this fibrotic process. Nevertheless, further studies are needed to establish the mechanisms of inflammatory cells recruitment around arteries and arterioles and to identify the links between hypertension and/or Ang II, and inflammatory cells.

**Acknowledgements** This work was supported by INSERM Unité 430 (Paris) and Université Paris VI (Paris). We thank Michel Paing for the photographs.

## References


1. Booz GW, Dostal DE, Singer HA, Baker KM (1994) Involvment of protein kinase C and $Ca^{2+}$ in angiotensin II-induced mitogenesis of cardiac fibroblasts. Am J Physiol 267:C1308
2. Brilla CG, Weber KT (1992) Reactive and reparative myocardial fibrosis in arterial hypertension in the rat. Cardiovasc Res 26:671–677
3. Brilla CG, Pick R, Tan LB, Janicki JS, Weber KT (1990) Remodling of the rat right and left ventricles in experimental hypertension. Circ Res 67:1355–1364
4. Brilla CG, Zhou G, Matsubara L, Weber KT (1994) Collagen metabolism in cultured adult rat cardiac fibroblasts: response to angiotensin II and aldosterone. J Mol Cell Cardiol 26:809–820
5. Cordell JL, Falini B, Erber WN, Ghosh AK, Abdulaziz Z, MacDonald S, Pulford KAF, Stein H, Mason DY (1984) Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). J Histochem Cytochem 32:219–229
6. Crabos M, Roth M, Hahn WA, Erne P (1994) Characterization of angiotensin II receptors in cultured adult rat cardiac fibroblasts: coupling to signaling systems and gene expression. J Clin Invest 93:2372
7. Diamond JR, Pesek-Diamond I (1991) Sublethal X-irradiation during acute puromycin nephrosis prevents late renal injury: role of macrophages. Am J Physiol 260:F779–F786
8. Dijkstra CD, Dopp EA, Joling P, Kraal G (1985) The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophages subpopulations in the rat recognized by monoclonal antibodies ED1, ED2, and ED3. Immunology 54: 589–599
9. Eng E, Véniant M, Floege J, Fingerle J, Alpers CE, Ménard J, Clozel J-P, Johnson RJ (1994) Renal proliferative and phenotypic changes in rats with two-kidney, one-clip Goldblatt hypertension. Am J Hypertens 7:177–185
10. Floege J, Alpers CE, Burns MW, Pritzl P, Gordon K, Couser WG, Johnson RJ (1992) Glomerular cells, extracellular matrix accumulation, and the development of glomerulosclerosis in the remnant kidney model. Lab Invest 66:485–497
11. Frederiksson K, Nordborg C, Kalimo H, Olsson Y, Johansson B (1988) Cerebral microangiopathy in stroke-prone spontaneously hypertensive rats. An immunohistochemical and ultrastructural study. Acta Neuropathol (Berl) 75:241–252
12. Gabe M (1968) Détection histochimique des principales activités enzymatiques. In: Gabe M (ed) Techniques histologiques. Masson, Paris, pp 601
13. Guan S, Fox J, Mitchell KD, Navar LG (1992) Angiotensin and angiotensin converting enzyme tissue levels in two-kidney, one clip hypertension rats. Hypertension 20:763–767
14. Helmchen V, Kneissler V, Bohle RM, Reher A, Groene HJ (1984) Adaptation and decompensation of intrarenal small arteries in experimental hypertension. J Cardiovasc Pharmacol 6:S696–S705
15. Hinglais N, Heudes D, Nicoletti A, Mandet C, Laurent M, Bariéty J, Michel J (1994) Co-localization of myocardial fibrosis and inflammatory cells in rats. Lab Invest 70:286–294
16. Hooke DH, Gee DC, Atkins RC (1987) Leucocyte analysis using monoclonal antibodies in human glomerulonephritis. Kidney Int 31:964–972
17. Johnson RJ, Alpers CE, Yoshimura A, Lombardi D, Pritzl P, Floege J, Schwartz SM (1992) Renal injury from angiotensin II-mediated hypertension. Hypertension 19:464–474
18. Jones CL, Buch S, Post M, McCulloch L, E. L, Eddy AA (1991) Pathogenesis of interstitial fibrosis in chronic purine aminonucleoside nephrosis. Kidney Int 40:1020–1031
19. Junqueira L (1979) Picrosirius staining plus polarizing microscopy: a specific method for collagen detection in tissue section. Histochem J 11:445–447
20. Korsgaard N, Mulvany MJ (1988) Cellular hypertrophy in mesenteric resistance vessels from renal hypertensive rats. Hypertension 12:162–167
21. Lan HY, Paterson DJ, Atkins RC (1991) Initiation and evolution of interstitial leukocytic infiltration in experimental glomerulonephritis. Kidney Int 40:425–433
22. Leibovich SJ, Ross R (1975) The role of the macrophage in wound repair. Am J Pathol 78:71–100
23. Loeb J, Bean B (1986) Antihypertensive drugs inhibit hypertension-associated aortic DNA synthesis in the rat. Hypertension 8:1135–1142
24. Mai M, Geiger H, Hilgers KF, Veelken R, Mann JFE, Dämmrich J, Luft FC (1993) Early interstitial changes in hypertension-induced renal injury. Hypertension 22:754–765
25. Matsubara H, Kanasaki M, Marasawa S, Tsukaguchi Y, Nio Y, Inada M (1994) Differential gene expression and regulation of angiotensin II receptor subtypes in rat cardiac fibroblasts and cardiomyocytes in culture. J Clin Invest 93:1592–1601
26. Ménard J, Catt KJ (1972) Measurement of renin activity concentration and substrate in rat plasma by radioimmunoassay of angiotensin I. Endocrinology 90:422–430
27. Michel JB, Dussaule JC, Choudat L, Auzan C, Nochy D, Corvol P, Ménard J (1986) Effects of antihypertensive treatment in one-clip, two kidney hypertension in rats. Kidney Int 29:1011–1020
28. Nagle RB, Kneisser MD, Bulger RE, Benditt EP (1973) Induction of smooth muscle characteristics in renal interstitial fibroblasts during obstructive nephropathy. Lab Invest 29:422427
29. Nicoletti A, Heudes D, Hinglais N, Appay MD, Philippe M, Sassy-Prigent C, Bariéty J, Michel J-B (1995) Left ventricular fibrosis in renovascular hypertensive rats: effect of losartan and spironolactone. Hypertension 26:101–111
30. Powell JS, Clozel JP, Müller RKM, Kuhn R, Hefti F, Hosang M, Baumgartner HR (1989) Inhibitors of angiotensin-converting enzyme prevent myointimal proliferation after vascular injury. Science 245:186–188
31. Sadoshima J, Izumo S (1993) Molecular characterization of angiotensin II-induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts: critical role of the AT(1) receptor subtype. Circ Res 73:413
32. Schorb W, Booz G, Dostal D, Conrad K, Chang K, Baker K (1993) Angiotensin II is mitogenic in neonatal rat cardiac fibroblasts. Circ Res 72:1245–1254

33. Shimamura T (1988) 11-Deoxycorticosterone-induced hypertension, glomerulosclerosis and renal arterial and arteriolar lesions. Jpn J Exp Med 58:225–228
34. Skalli O, Ropraz P, Trzeciak A, Benzonana G, Gillessen D, Gabbiani G (1986) A monoclonal antibody against α-smooth muscle cell actin: a new probe for smooth muscle differentiation. J Cell Biol 103:2787–2796
35. Truong LD, Farwood A, Tasby J, Gillum D (1992) Experimental chronic renal ischemia: morphologic and immunologic studies. Kidney Int 41:1676–1689
36. Van Goor H, Van der Horst MLC, Fidler V, Grond J (1992) Glomerular macrophage production affects mesangial expansion in the rat after renal ablation. Lab Invest 66:564–571
37. Villarreal FJ, Dillmann WH (1992) Cardiac hypertrophy-induced changes in mRNA levels for TGF-β1 fibronectin, and collagen. Am J Physiol 262:H1861–H1866
38. Weisstuch JM, Dworkin LD (1992) Does essential hypertension cause end-stage renal disease? Kidney Int 41:S-33–S-37
39. Wiggins R, Goyal M, Merritt S, Killen PD (1993) Vascular adventitial cell expression of collagen I messenger ribonucleic acid in antiglomerular basement membrane antibody-induced crescentic nephritis in the rabbit. A cellular source for interstitial collagen synthesis in inflammatory renal disease. Lab Invest 68:557–565
40. Williams AF, Galfre G, Milstein C (1977) Analysis of cell surfaces by xenogeneic myeloma-hybrid antibodies. Differentiation antigens of rat lymphocytes. Cell 12:633–673
41. Wilson C, Byrom FB (1939) Renal changes in malignant hypertension. Experimental evidence. Lancet II:136–139
42. Wolf G, Neilson EG (1993) Angiotensin II as a hypertrophogenic cytokine for proximal tubular cells. Kidney Int 43:S100–S107
43. Xu Y, Arnal JF, Hinglais N, Appay M-D, Laboulandine I, Bariéty J, Michel JB (1995) Renal hypertensive angiopathy. Comparison between chronic NO suppression and DOCA-salt intoxication. Am J Hypertens 8:167–176
44. Zhou G, Brilla CG, Weber KT (1992) Angiotensin II-mediated stimulation of collagen synthesis in cultured cardiac fibroblasts. FASEB J 6:A1914