To elucidate the mechanisms of hypertensive renal injury, we investigated the time course and extent of changes in matrix composition, as well as cell proliferation and infiltration in two-kidney, one clip rats. The nonclipped kidneys from hypertensive and sham-operated control rats (n=5 to 10 in each group) were studied at 7, 14, 21, and 28 days after clipping. Systolic blood pressure was elevated by day 7 (154±3 versus 111±4 mm Hg in sham group, P<.001, n=10 each). Hypertension resulted in an early expansion of the interstitial volume by 37%, whereas hypertensive vascular changes and glomerular injury did not become evident until day 21. Immunofluorescence studies revealed an early interstitial accumulation of collagens I, III, IV, V, VI, and fibronectin by day 7. In contrast, the glomeruli showed a mild to moderate increase in collagens I, III, IV, V, laminin, and fibronectin but not collagen VI later in the established phase of hypertension. Staining for proliferating cell nuclear antigen as a marker of cell replication was increased in tubular epithelial but not interstitial or glomerular cells. A progressive infiltration of macrophages (16±2 versus 9±1 ED1+ cells/mm², P<.05, n=6) and T lymphocytes (93±15 versus 74±7 CD4+ cells/mm², n=8) in the cortical interstitium had already occurred by day 7. On the other hand, only macrophages increased in number within the glomeruli. Thus, renovascular hypertension leads to an early tubular cell proliferation, mononuclear cell recruitment, and deposition of matrix proteins primarily within the interstitium. We conclude that the injury producing nephrosclerosis in this model extends far beyond the glomeruli. Both the tubules and the interstitium are actively involved and may be the more important initial sites of injury. (Hypertension. 1993;22:754-765.)

KEY WORDS • hypertension, renovascular • nephrosclerosis • extracellular matrix • macrophages • T lymphocytes, helper-inducer

Hypertensive nephrosclerosis (HNS) is one of the most important causes of end-stage renal failure.1 HNS may by itself accelerate and maintain high blood pressure4 and thereby lead to a vicious circle of hypertension and renal injury.5 The cellular and molecular mechanisms leading to HNS are still poorly understood. Vascular changes have been implicated as primary features of HNS; however, glomerular and interstitial alterations have been described as well.4-8

Recent studies have provided considerable insight into the molecular and cellular mechanisms causing inflammatory renal injury, in particular with respect to glomerular damage.5-11 Some authors have advanced the notion that proliferation of resident cells and mediators, such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), plays a key role in the development of glomerulonephritis.12-17 The mechanisms involved in hypertensive renal injury are much less clear.

Studies on HNS during the past decade have focused on hypertensive damage to glomeruli.18-22 However, interstitial fibrosis may be an even more important determinant of renal injury. This hypothesis was originally raised by Bohle et al18,23 and has recently found increased recognition.24 To gain insight into mechanisms, we examined cell proliferation, participation of mononuclear cells, and accumulation of extracellular matrix molecules during the development of HNS. We focused on the tubulointerstitial and examined changes early after the induction of hypertension. We used the contralateral, nonclipped kidney of two-kidney, one clip (2K1C) Goldblatt renovascular hypertensive rats as a model of HNS.5,21 Our results draw attention to cell proliferation, matrix deposition, and the participation of lymphocytes and macrophages at extraglomerular sites.

Methods
Animal Model and Experimental Design

Renovascular hypertension was produced by the 2KIC method in male Sprague-Dawley rats (SAVO, Kissleg, FRG) (weight, 150 to 180 g) by placing a 0.20-mm i.d. silver clip around the left renal artery through a flank incision while the rats were under methohexital anesthesia. The right kidney remained...
Fig 1. Line graphs show time course of systolic blood pressure (panel A) and kidney weight (panel B) (mean±SEM). A shows that, from day 3, the values in the two-kidney, one clip (2K1C) hypertensive group are significantly different (*P<.05, **P<.005, ***P<.0005) vs the time-matched, sham-operated control (sham) group; B, from day 14, the unclipped kidney weights are significantly higher (#P<.01, ##P<.002) in the 2K1C group compared with the time-matched, sham-operated control group.

untouched. A similar procedure without clip application was performed in sham-operated control rats. The rats were allowed free access to standard chow (Altromin, Lage, FRG) and tap water. Systolic arterial blood pressure was measured under light ether anesthesia by tail-cuff plethysmography. Measurements were performed, without knowledge of the regimen, weekly and on the day before the rats were killed. Body weight was measured twice weekly. Rats that failed to thrive or gain weight were excluded because these phenomena may herald the occurrence of malignant hypertension.25

The right, nonclipped kidneys from 2K1C or sham-operated rats were removed after 1, 2, 3, and 4 weeks (n=5 to 10 each). Blood samples for measurement of serum urea were obtained from the eye while the rats were under light ether anesthesia. For determination of plasma renin activity (PRA), blood was obtained from a catheter inserted in the femoral artery in additional groups of conscious rats. The rats were anesthetized with methohexital and exsanguinated. The hearts and kidneys were removed and weighed. The kidneys were bisected along their longitudinal axis for morphological and immunohistological examination. Serum urea was measured by automated techniques, and PRA was determined by radioimmunoassay.

All experimental procedures were performed in accordance with the guidelines of the American Physiological Society and were approved by the local government's ethics committee (Regierung von Mittelfranken, AZ 211-2531.3-4/90).

Renal Morphology

For light microscopy, one half of each kidney was fixed in 10% buffered Formalin and embedded in paraffin. Three-micrometer-thick sections were stained with the periodic acid-Schiff reagent, hematoxylin and eosin, van Gieson's elastica, and the movat silver technique. All histological studies were performed without knowledge of the regimens or their duration (blinded). The extent of the arteriolar and glomerular changes were determined by grading 100 arterioles and 200 glomeruli from each tissue section. The mean value was calculated for each rat. Arteriosclerotic changes were graded as IV in each experimental animal if more than 40% of the arterioles were obliterated. The glomerulosclerosis score was graded as IV if more than 10% of the glomeruli were completely scarred. Grading was performed as described by Bader et al26 according to a semiquantitative score of 1 to 4 as follows.

Arterioles. 1, No changes; 2, hyalinosis of the arteriolar wall up to 50% of its circumference; 3, hyalinosis of

<table>
<thead>
<tr>
<th>Table 1. Organ Weights and Renal Biochemical Parameters</th>
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<tbody>
<tr>
<td>Body Weight, g</td>
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<td>---------------</td>
</tr>
<tr>
<td>Day 7</td>
</tr>
<tr>
<td>Sham</td>
</tr>
<tr>
<td>2K1C</td>
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<tr>
<td>Day 28</td>
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<tr>
<td>Sham</td>
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<td>2K1C</td>
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Sham indicates sham-operated normotensive control group; 2K1C, two-kidney, one clip hypertensive group; PRA, plasma renin activity; Left, clipped kidney; Right, contralateral, nonclipped kidney; and AngⅠ, angiotensinⅠ. Values are mean±SEM.

*P<.01, †P<.05, ‡P<.001 vs time-matched, sham-operated control group.
the wall between 50% and 100% of its circumference but without narrowing of the lumen; 4, complete hyalnosis of the wall with luminal encroachment.

Glomeruli. 1, Normal glomerulus; 2, slight glomerular damage with mild mesangial expansion and increased matrix; 3, moderate mesangial expansion and increased matrix, possibly accompanied by mild proliferation of visceral epithelial or mesangial cells with hyalin droplets; 4, focal, segmental, or global sclerosis and/or hyalnosis up to complete scarring of the glomerulus.

Interstitium. The volume was estimated by morphometric methods by means of a point-counting technique as described by Weibel and Bennett et al. Sections were examined with a 100-point grid fitted in the eyepiece of the microscope. Twenty consecutive high-power fields (×400) were counted in all cases to evaluate the extent of the interstitial fibrosis.

Matrix Molecules

The immunofluorescence of the matrix proteins collagens I, III, IV, V, VI, laminin, and fibronectin was examined after renal tissue was snap-frozen in isopentane, precooled in liquid nitrogen, and stored at −70°C. Five-micrometer-thick cryostat sections were placed on chromalaun/gelatin-coated slides, air-dried, and processed by an indirect immunofluorescence technique to detect the matrix components.

After preincubation with 100% fetal calf serum (FCS) for 30 minutes, the sections were consecutively covered with the specific primary polyclonal antibodies diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) for 1 hour, and with a secondary fluorescein-isothiocyanate-conjugated immunoglobulin (Ig) G [sheep anti-rabbit F(ab), fragment (1:10, Boehringer Mannheim, FRG) or donkey anti-goat IgG (1:40, Dianova, FRG)] diluted in PBS containing 1% BSA and 1% FCS for 60 minutes. The incubations were carried out at room temperature in a humid chamber. Each of these steps was preceded by three washes in PBS for 5 minutes. The sections were mounted in buffered moviol/gelatin and examined with a Leitz fluorescent microscope.

To assess matrix accumulation, we compared the immunofluorescence staining of kidneys from the 2K1C group with those from the sham-operated group. A semiquantitative estimate reflects changes in the area of matrix staining rather than intensity. The sections were independently graded by three observers unaware (blinded) of the regimens.

Mononuclear Infiltration

For immunohistochemical staining of mononuclear cell infiltration, 5-μm-thick cryostat sections were fixed briefly in cold acetone, air-dried, immersed in Tris-buffered saline (TBS, pH 7.4), and preincubated with 100% FCS for 30 minutes at room temperature. Monoclonal anti-ED1 or anti-CD4 antibodies diluted in 1% BSA/TBS were applied for 60 minutes at room temperature. After washing with TBS (three times for 5 minutes), the sections were incubated with rabbit antimouse immunoglobulin, and the immunoreactivity was visualized by an alkaline phosphatase—anti-alkaline phosphatase (APAAP) method (Dakopatts, FRG) as proposed by the manufacturer using the neufuchsin substrate kit (Dakopatts) for detection. The endogenous alkaline phosphatase is blocked by addition of levamisole (Sigma, FRG) at an end concentration of 10 mmol/L to the substrate solution. The sections were light counterstained in hematoxylin for 10 seconds (Gill No. 3, Sigma), blued in running tap water (10 minutes), and mounted with aquatex (Merck, FRG).
The quantitation of the tubulointerstitial infiltration was determined by selecting cortical areas at random. Large blood vessels and glomeruli were avoided. The numbers of ED1+ and CD4+ cells were counted in 20 high-power sections (×250 and ×400) by means of a 1-cm² grid fitted into the eyepiece of the microscope. The mean values are expressed as cells per millimeter squared ±SEM. The mean numbers of ED1+ and CD4+ cells per glomerular cross section were determined by evaluating 50 glomeruli in each renal section.

**Cell Proliferation**

The immunostaining for proliferating cell nuclear antigen (PCNA) was carried out on methyl Carnoy’s fixed and paraffin-embedded tissues. Briefly, the sections (3-μm thick) were deparaffinized in xylene and a graded series of ethanols and rehydrated three times for 3 minutes in PBS (containing 0.01% Triton X-100 plus 0.1% BSA). The sections were then covered with the primary monoclonal PCNA (PC10) antibody diluted in PBS overnight at 4°C in a humid chamber, washed with PBS (0.01% Triton X-100 plus 0.1% BSA), and subsequently processed using the APAAP method (Dako-patts) as above.

Different PCNA staining patterns are believed to reflect individual phases of the cell cycle. The distinct and intense 2+ and 3+ nuclear staining identifies cells in the S phase. The cell proliferation was quantified as...
### Table 2. Immunohistochemistry of Extracellular Matrix (Staining Compared With Sham-Operated Rat Renal Tissue)

<table>
<thead>
<tr>
<th></th>
<th>Collagen Type I</th>
<th>Collagen Type III</th>
<th>Collagen Type IV</th>
<th>Collagen Type V</th>
<th>Collagen Type VI</th>
<th>Fibronectin</th>
<th>Laminin</th>
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<td>Mesangium</td>
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<td>→</td>
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<td>GBM</td>
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<tr>
<td>Bowman’s capsule</td>
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<tr>
<td>Interstitium</td>
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<td>TBM</td>
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<td>Vessels</td>
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<td>(†) (†)</td>
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<td>(†) ↑ (†)</td>
<td>(†) (†)</td>
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</table>

Early indicates early hypertensive phase (1 to 2 weeks); Late, later hypertensive phase (3 to 4 weeks); GBM, glomerular basement membrane; TBM, tubular basement membrane; -, not detectable; →, no change; †, mild increase; ††, moderate increase; and †††, marked increase. Accumulation of extracellular matrix molecules during the development of hypertension. Each score reflects changes in the extent rather than intensity of matrix staining.

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**Fig 4.** Immunofluorescence staining of matrix proteins. Vertical axis shows Sham, day 14, and day 28 sections. Horizontal axis shows collagens (Col) I and IV (above) and VI and fibronectin (facing page). Increased staining over time, earlier and more prominent in the interstitium, later and less so in the glomeruli, is observed (original magnification ×250).
follows. We counted only cells with intense, deep red
3+ nuclear staining. The mean number of proliferating
cells in the tubulointerstitium was determined by count-
ing the PCNA+ cells in 30 fields per tissue section at a
magnification of ×250. We used a 1-cm² grid for this
purpose that was fitted to the eyepiece of the micro-
scope and ignored large blood vessels and glomeruli.
The glomerular PCNA+ cells were counted separately
by sequentially examining 90 glomeruli in each kidney
section.

Antibodies
The following polyclonal and monoclonal primary
antibodies were used: (1) collagen I, rabbit anti-rat
antiserum (1:10, Pasteur Diagnostica, FRG); (2) colla-
gen III, rabbit anti-rat antiserum (1:20, Pasteur Diag-
nostica), both of which were purified on a protein
A-sepharose column (low-salt method); (3) collagen IV,
purified rabbit anti-mouse IgG (1:200, Collaborative
Research Inc, Bedford, Mass); (4) collagen V, affinity
purified goat anti-human and bovine antibodies (1:30,
Southern Biotechnology Assoc, Inc), which was cross-
absorbed against all irrelevant collagen types to remove
cross-reacting moieties; (5) collagen VI, rabbit anti-
human antiserum (1:75, Heyl, FRG); (6) fibronectin,
goat anti-human IgG (1:1000, gift from Dr S. Goodman,
Erlangen, FRG); (7) laminin, rabbit antiserum against
mouse laminin prepared from EHS-tumor according to
Timpl et al30 (1:100, gift from Dr J. Mollenhauer,
Chicago, Ill); (8) ED1, (1:1000, Serotec, FRG), a mu-
rine monoclonal anti-rat IgG to a cytoplasmic antigen
present in macrophages, monocytes, and dendritic cells;
(9) W3/25, (1:2000, Serotec), a murine monoclonal
anti-rat IgG to a surface CD4 antigen on T lymphocyte
helper cells; and (10) PC10 (1:100, Dianova, FRG), a
murine monoclonal IgG against recombinant rat
PCNA.31,32 The proliferating cell nuclear antigen
PCNA/cyclin is a highly conserved 36 kD auxiliary
protein of DNA polymerase delta,33,34 Synthesis of this
protein begins in the late G1 phase and peaks during
the S phase, followed by a decrease in G2/M phase of
the cell cycle.29,35 Negative controls consisted of substi-
tution of the primary antibody with both equivalent concentrations of an irrelevant murine monoclonal antibody or normal rabbit IgG and PBS.

**Statistical Analysis**

The data are expressed as mean±SEM. Significance of differences between 2K1C and sham groups was assessed by the nonparametric Mann-Whitney U test. A value of \( P<.05 \) was considered significant. To correlate interstitial volume with blood pressure and PRA, we used the Spearman’s rank correlation coefficient.

**Results**

Blood pressure, body and organ weights, serum urea concentrations, and PRA are shown on Fig 1 and Table 1, respectively. Mean systolic blood pressure was elevated in 2K1C rats by day 3 and increased further to 224±12 mm Hg thereafter. Control rats had no change in blood pressure. At day 28, the 2K1C rats weighed less and had a greater heart and nonclipped right kidney-to-body weight ratio than sham controls. The change in kidney weight not factored for body weight is shown in Fig 1B. The nonclipped kidney in 2K1C rats increased significantly and progressively in size compared with sham controls. PRA value was significantly elevated at day 7 and increased even more by day 28. Serum urea was unchanged in the early phase and was significantly higher than controls at day 28.

**Morphological Studies**

Semiqualitative, morphological alterations in the nonclipped kidney of 2K1C rats are shown in Fig 2. The arterioles showed arteriosclerotic changes (Fig 2A). The alterations progressed to 50% hyalinosis of the circumference (stage 2), to hyalinosis between 50% and 100% (stage 3), to luminal obliteration (stage 4) by days 14, 21, and 28, respectively. The glomerular damage showed a similar time course. On these same days, mild (stage 2) to moderate (stage 3) mesangial expansion and matrix increase, and focal segmental and global sclerosis (stage 4) were observed (Fig 2B). Panel C illustrates the progressive interstitial expansion by 17% at day 7 to 156% at day 28, which is accompanied by initial focal to widespread tubulus atrophy as well as dilatation. Six representative light microscopic sections of vascular, glomerular, and tubulointerstitial alterations are shown in Fig 3. Interstitial volume correlated with blood pressure \( (r=.74, P<.002, n=15) \) and PRA \( (r=.62, P<.02, n=14) \) in 2K1C but not in sham control rats.

**Immunofluorescence of Extracellular Matrix Molecules**

Hypertension-induced changes in extracellular matrix molecules are summarized in Table 2. The time-dependent changes are expressed in terms of early (days 7 and 14) and late (days 21 and 28). The most consistent changes were observed in the renal interstitium. Here a mild, focal accumulation of collagens I, II, IV, V, VI, and fibronectin was already detectable in the early hypertensive phase. These accumulations were generalized and more pronounced during the late phase and paralleled the expansion in interstitial volume. In contrast, the staining pattern of the glomerular matrix remained unchanged in the early phase of hypertension. Mild to moderate increase in glomerular staining was observed during the late phase for collagens I, III, IV, V, fibronectin, and laminin, whereas the immunofluorescent staining of collagen VI was unchanged. Representative examples are shown in Fig 4. Sections from shams, day 14, and day 28 are shown on the vertical axis. On the horizontal axis are shown collagen I, collagen IV, collagen VI, and fibronectin. Glomeruli, occasional blood vessels, and interstitium can be identified. The striking interstitial changes and milder glomerular changes are evident.

**Localization of Early Cell Proliferation**

Immunostaining with PCNA was used to identify cell proliferation. We observed a proliferation of tubular epithelial cells in the nonclipped kidney of 2K1C rats (Fig 5). By day 14 (Fig 5A), 44±14 PCNA+ tubular cells were seen, compared with 1±0.6 PCNA+ tubular cells \( (P<.05) \) in sham control rats (mean value of 30 counted fields per section, \( n=5 \) each). On the other hand, PCNA+ cells in the interstitium itself did not increase significantly (Fig 5B). An intense PCNA+ staining was most prominent in tubules showing atrophy, whereas healthy-appearing tubules showed less prominent PCNA+ staining. Significant proliferation of interstitial cells could be detected only at day 28 when the tubular proliferation was decreased. In contrast, the glomeruli (Fig 5C) showed a modest increase in staining, which reached statistical significance in the early hypertensive phase. PCNA+ staining was not observed in blood vessels (45 vessels were evaluated).

**Early Interstitial Mononuclear Infiltration**

The progression of tubulointerstitial injury was associated with an early significant increase in interstitial infiltration by T helper cells (Fig 6A). The values for CD4+ cells were 262±32 versus 96±16 cells/mm² at day 14 \( (P<.005, n=8) \) and 355±30 versus 118±29 cells/mm² \( (P<.005, n=8) \) at day 28. A significant increase in the influx of macrophages was also observed (Fig 6B). These values for ED1+ cells were 26±3 versus 12±1 cells/mm² at day 14 \( (P<.005) \) and 39±12 versus 14±1 cells/mm² \( (P<.005, n=8) \) at day 28. In the glomeruli, no change for CD4+ cells could be identified (Fig 6C), but a mild infiltration of ED1+ cells was observed in the early phase (Fig 6D). At day 14, the values were 0.6±0.09 versus 0.23±0.03 cells per glomerulus \( (P<.005, n=8) \).

Representative PCNA+ sections can be seen in Fig 7. The sham section is shown in Fig 7a. At day 14 (Fig 7b), a marked increase in proliferation of tubular but not glomerular or interstitial cells can be identified. The staining for CD4+ and ED1+ cells can be seen in Fig 8. Control sections are shown on the left, experimental sections at day 14 on the right. Fig 8b represents the early increased infiltration of CD4+ cells in the interstitium and Fig 8d the influx of ED1+ cells and their perivascular localization.

**Discussion**

Our study was designed to elucidate mechanisms involved in the development of HNS in the nonclipped kidney of 2K1C rats. The main findings were an early and progressive accumulation of fibronectin and collagens I, III, IV, V, and VI in the renal interstitium. This accumulation occurred in parallel with the expansion of
the interstitial volume. Changes in glomerular extracellular matrix (ECM) became evident later during the course of HNS. Evidence for proliferation was detected in tubular epithelial but not glomerular cells. In addition, infiltration of T helper cells and macrophages into the renal interstitium was also an early event in the progression of nephrosclerosis. These findings draw attention to the importance of interstitial changes, as well as to the involvement of mononuclear cells in a noninflammatory, pressure-induced model of renal injury.

The 2K1C Goldblatt model is a well-established experimental model of renovascular hypertension.36 The pathophysiology of the hypertension is far more complicated than Goldblatt could have realized and has recently been reviewed.36 Numerous studies in the nonclipped kidney exposed to the high blood pressure have shown altered hemodynamics and development of vascular and glomerular lesions, such as fibrinoid necrosis of small arterioles and focal glomerulosclerosis.5,6,21

Intraglomerular hypertension and hyperfiltration were discussed as important hemodynamic factors in the progression of glomerular injury.36 Some hypertensive models, such as spontaneously hypertensive rats, are relatively resistant to the development of glomerulosclerosis, presumably due to afferent arteriolar vasoconstriction.22,29 On the other hand, in Dahl salt-sensitive, deoxycorticosterone acetate-salt, or renal ablation models with preglomerular dilatation, glomerulosclerosis is severe.18-20 Micropuncture studies in the nonclipped kidney of 2K1C rats have revealed glomerular hypertension and hyperfiltration despite some degree of afferent arteriolar constriction.34,21 The calcium antagonist nitrrendipine, which reduced preglomerular resistance, accelerated the progression of glomerulosclerosis.40

Although the glomeruli and glomerular hemodynamics have received the greatest degree of attention in this model and in most others, tubulointerstitial injury,8-23-41 abnormal regulation of extraglomerular cell growth,42-44 and altered collagen synthesis45 and degradation46 or both may play an equally important role. The renal interstitium is more than a passive space in which the truly functional units reside. Rather, it is a highly dynamic tissue in which structural support is rendered, fibers and ground substance are produced and degraded, substance exchange and lymphatic drainage are provided, and hormonal functions are conducted.47 Interstitial fibrosis occurs in all renal diseases, including glomerulonephritis,23-41 cystic disease,46 renal ablation,49 diabetes,50 and hypertension-induced nephrosclerosis.44 Whether interstitial fibrosis is a primary event or rather is secondary to glomerular damage is currently discussed.24 Kuncio et al24 have proposed that recruitment of inflammatory cells, cytokines, proliferation of fibroblasts, and matrix deposition may be key events in the fibrotic process. However, no clear evidence to support their notion has been reported.

In contrast to our knowledge about collagen deposition in glomerular disease,9-51 information about the involvement of ECM components during the course of tubulointerstitial disease is rudimentary. Recent studies revealed an interstitial involvement of collagens I, III, and IV in crescentic glomerulonephritis,23-41 cystic disease,46 renal ablation,49 diabetes,50 and hypertension-induced nephrosclerosis.44 Whether interstitial fibrosis is a primary event or rather is secondary to glomerular damage is currently discussed.24 Kuncio et al24 have proposed that recruitment of inflammatory cells, cytokines, proliferation of fibroblasts, and matrix deposition may be key events in the fibrotic process. However, no clear evidence to support their notion has been reported.

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Tubulo-interstitium

Fig 6. Bar graphs show quantification of mononuclear infiltration. Early progressive and significant increases in T helper (CD4+) cells (panel A) and macrophages (panel B) were seen in the cortical interstitium from the two-kidney, one clip (2K1C) hypertensive group (mean±SEM). On the other hand, only macrophages (ED1+ cells) (panel D) increased in number in the glomeruli. *P<.05, #P<.005 vs time-matched, sham-operated control (sham) group.

changes appeared to be unrelated to the glomerular alterations, which were lesser in extent and occurred later in the course of HNS.

In our hypertensive model, intraglomerular increase in the ECM components collagens I, III, IV, V, laminin, and fibronectin could be identified, whereas collagen VI remained unchanged. This ECM pattern is similar to those described in other glomerular diseases, such as proliferative glomerulonephritis,9-52 remnant kidney, 31 and diabetic glomeruli.53

The deposition of ECM is one of the more complex and highly variable components of the tubulointerstitial fibrosis. The nature of the response is dependent on a host of different factors and on the target cells that express the fibrotic matrix.24 The role of fibroblasts in interstitial nephritis has been emphasized.42,43,45 Rodeumann and Müller,42 Müller and Rodeumann,43 and Müller et al46 found that fibroblasts from human kidneys with interstitial fibrosis exhibited enhanced growth, a prolonged mitotic lifespan, and more actively produced collagens I, III, V, and fibronectin compared with fibroblasts from normal human kidneys. However, we could not find any evidence for proliferation of interstitial cells.

Proximal tubular epithelial cell lines are known to secrete collagens I, III, IV, V, procollagens, and laminin.57 Furthermore, epithelial cells from rat kidney have been shown to secrete collagens I and III; the production can be upregulated by growth factors.58 The induction of fibrogenesis is likely to involve a variety of growth factors,59 as well as cytokines such as TGF-β,61,60,61 and PDGF.12,13,44 Johnson et al44 studied renal injury in hypertension induced by infusion of Ang II and found that PDGF β-chain mRNA could be localized at the site of interstitial injury. The potential cytokine source could be either "stimulated" tubulointerstitial cells52,62 and/or infiltrating mononuclear cells, such as macrophages64 and T lymphocytes, in particular T helper cells.65

In support of such mechanisms, we also observed focal tubular atrophy and dilation, tubular cell proliferation, and the appearance of macrophages and T lymphocytes in the hypertensive kidneys early in the development of tubulointerstitial fibrosis. We found significant proliferation of tubular cells primarily in areas of tubular injury, whereas glomerular and interstitial or vascular cell proliferation was minimal or absent. In parallel, we observed focal infiltration of T helper cells and a perivascular influx of macrophages after 1 week of 2K1C hypertension. This infiltration spread to the interstitium during the following weeks. In various forms of glomerulonephritis an abnormal expression of myosin heavy chain-class II antigens66 and of ICAM-167 was observed on renal tubular epithelial cells, providing a mechanism for the direct involvement of antigen presentation to T lymphocytes. Only a small infiltration of macrophages could be documented in glomeruli. In contrast, an early glomerular cell proliferation was described in the remnant kidney model. The authors of this report proposed that proliferation and macrophage influx in glomeruli may lead to an increase in ECM proteins.51

In contrast to our findings, proliferation of vascular smooth muscle and interstitial cells was described by Johnson et al64 in Ang II–induced hypertension. These authors noted that the cell proliferation could be related to the presence of angiotensin, the presence of hypertension, or a combined action of both. Ang II is probably not a mitogen for vascular smooth muscle cells in vitro64; however, it induces vascular smooth muscle cell proliferation in vivo.44,69 This response may be due to cofactors such as elevated blood pressure or stimulation of sympathetic outflow.70 Although Ang II is
clearly involved in the pathogenesis of 2K1C hypertension, we did not observe vascular smooth muscle cell proliferation. Our results are in agreement with those of others who examined extrarenal vessels of 2K1C hypertension.71 The differences between our findings and those of Johnson et al.44 may be related to differences in the hypertensive models. Increased levels of Ang II are not the sole pathogenetic factor in 2K1C hypertension. In fact, it is not clear whether the nonclipped kidney of the 2K1C model is exposed to elevated concentrations of Ang II. Circulating Ang II is elevated; however, renin mRNA is locally suppressed in the unclopped kidney.72-73 Therefore, the real "net" effect of Ang II or its actual concentration in the contralateral kidney of the 2K1C model is not known.

The measured positive correlations of interstitial volume with PRA and blood pressure suggest that the renin-angiotensin system may play a role in development of the interstitial changes. Ang II could contribute to tubular cell proliferation or hypertrophy74 as well as to increased matrix synthesis.75 The blood pressure per se may also have an effect by mechanical force and stretch. The results of an in vitro study suggest that capillary expansion and mesangial stretching per se can stimulate ECM production of collagens I, IV, laminin, and fibronectin.76

**Fig 7.** Photomicrographs of the proliferating cell nuclear antigen (PCNA) immunostaining show sections from control tissue (panel a) and from an unclipped kidney of a two-kidney, one clip rat at day 14 (panel b). The prominent PCNA+ cells are tubular rather than interstitial or glomerular cells (magnification ×250).

**Fig 8.** Mononuclear infiltration. Left panels show staining for CD4+ (panel a) and ED1+ (panel c) cells in control tissue. Right panels show a widespread CD4+ infiltration in the interstitium (panel b) and a primarily perivascular localization of ED1+ cells (panel d) after 2 weeks hypertension (magnification ×250).
Taken together, our results emphasize the importance of tubulointerstitial injury in the progression of HNS. The early occurrence of interstitial injury excludes the possibility that these changes are secondary to glomerular lesions. Mononuclear cells and their released mediators may support the fibrogenesis. The initial stimulus for the infiltration is unknown. Hypertension-induced, vascular endothelial lesions may be a trigger in this process.

In summary, our results suggest that the mechanisms of hypertension-induced renal injury in 2K1C hypertension must be reassessed to include a primary role for hypertension-induced renal injury in 2K1C hypertension.

The consequences of tubulo-interstitial changes for renal function must be reassessed to include a primary role for hypertension-induced renal injury in 2K1C hypertension.

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