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Age-related glomerulosclerosis and interstitial fibrosis in Milan normotensive rats: A podocyte disease

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Age-related glomerulosclerosis and interstitial fibrosis in Milan normotensive rats: A podocyte disease. In Milan normotensive (MNS) rats glomerulosclerosis and interstitial fibrosis develop spontaneously in the absence of hypertension. Renal changes were sequentially assessed in these rats between 2 and 10 months of age. At 10 months, rats were characterized by heavy proteinuria, increased serum creatinine, focal or global glomerulosclerosis in 51 ± 12% of the glomeruli as well as tubulointerstitial injury involving > 25% of the section area. Cell injury in podocytes (evidenced as increased expression of desmin and by electron microscopy) and interstitial fibroblasts (increased expression of α-smooth muscle actin) and mild glomerular hypertrophy were witnessed as early as three to four months of age and preceded glomerulosclerosis and interstitial fibrosis. Only minor evidence of mesangial cell activation (as assessed by glomerular *de novo* α-smooth muscle actin or type I collagen expression or increased cell proliferation) was noted throughout the observation period. Later stages of the disease were characterized by glomerular and/or tubulointerstitial macrophage influx and osteopontin expression (a chemoattractant), mild accumulation of lymphocytes, platelets, fibrinogen, as well as by a progressive accumulation of various matrix proteins. Progressive renal disease in MNS rats is thus noteworthy for the relative lack of mesangial cell activation. Rather, early podocyte damage, induced by yet unknown mechanisms, may underlie the development of glomerulosclerosis and subsequent interstitial fibrosis.

Progressive renal dysfunction even after the apparent clinical resolution of an injurious condition is common in many human glomerular diseases [1]. Several factors have been identified which contribute to the progression of renal dysfunction, including the presence of systemic hypertension, glomerular hyperfiltration and/or hypertrophy, and hypercholesterolemia [1–4]. Histologically, most progressive renal diseases are characterized by the development of glomerulosclerosis and secondary renal interstitial fibrosis [4, 5]. Based on this latter observation, it has been proposed that various types of renal injury, for example, immune-mediated, hypertensive or toxic injury, can enter a final common pathway leading to end-stage renal failure [1–5].

A variety of experimental models has been used to study the changes that precede or parallel the development of glomerulosclerosis and renal interstitial fibrosis [6–11]. Several previous studies [6, 9, 10, 12–15] have analyzed the immunohistochemical changes that occur in the kidney during damage induced by angiotensin II and/or hypertension, including angiotensin II infused rats, the two-kidney-one-clip model, and the 5/6 nephrectomy model. These studies have demonstrated that: (1) Both glomerular mesangial cells and interstitial fibroblasts may acquire characteristics of myofibroblasts during progressive renal damage [6, 9, 11, 13, 14]; (2) glomerular cell proliferation, possibly mediated by platelet-derived growth factor (PDGF), as well as augmented tubulointerstitial cell proliferation may be detected early during the course of the disease models [6, 9, 13]; and (3) progressive infiltration of macrophages in glomeruli and the tubulointerstitium accompanies the development of proteinuria, renal dysfunction, matrix accumulation, glomerulosclerosis and interstitial fibrosis [9, 10, 12–15].

In Milan normotensive (MNS) rats, marked glomerulosclerosis and interstitial fibrosis develop spontaneously with age in the absence of systemic hypertension [16]. We recently reported that an angiotensin converting enzyme inhibitor, captopril, did not ameliorate the development of proteinuria or glomerulosclerosis in these rats, when given at a dose that did not affect systemic blood pressure but which led to a twofold increase of plasma renin activity [17]. Thus, in contrast to the angiotensin II infusion, two-kidney-one-clip, and the 5/6 nephrectomy models, neither hypertension nor angiotensin II appear to be of central importance in the development of progressive renal dysfunction in MNS rats. We have therefore conducted the present study to evaluate the renal changes preceeding overt parenchymal damage and whether, despite the aforementioned differences, similar changes as outlined above accompany the development of progressive renal dysfunction in MNS rats.

Methods

Experimental design

MNS rats, originally obtained from Dr. G. Bianchi (Milan, Italy), have been bred in the animal facilities of the Hannover Medical School for the last 10 years. The experimental design was approved by the local animal protection committee. Four male rats each were studied at the ages of 2, 3, 4, 5, 6, 7, 8, and 10 months. Since the MNS rats are genetically related to Wistar rats [16], five male Wistar rats (obtained from the animal facilities of

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the Hannover Medical School) aged two months and five Wistar rats aged 10 months served as controls. In each rat a 24-hour urine collection was performed to determine proteinuria, and a serum sample was obtained and stored at −20°C for the measurement of serum creatinine. After this rats were anesthetized by an intraperitoneal injection of 10 mg ketamine/100 g body wt (WDT, Hannover, Germany) and 2 mg xylazine per animal (Bayer, Leverkusen, Germany) and mean arterial blood pressures were measured directly in the right carotid artery using a Statham transducer (Hellige Instruments, Freiburg, Germany). Rats were then sacrificed by decapitation. After sacrifice, kidneys were quickly removed, a 3 to 4 mm thick longitudinal section of the left kidney was obtained for immunohistology (see below) and the right kidney was weighed. Renal tissue was studied to assess glomerular and tubulointerstitial injury, cellular expression of α-smooth muscle actin and/or desmin, cell proliferation (as defined by staining for the proliferating cell nuclear antigen, PCNA), infiltrating inflammatory cells (monocytes/macrophages, neutrophils, lymphocytes, platelets), fibrinogen, chemoattractants (osteopontin), and extracellular matrix proteins (types I and IV collagen, laminin, and fibronectin).

To further analyze the very early renal changes in MNS rats, an additional 11 rats aged 10 to 19 weeks were investigated by electron microscopy.

Renal morphology

Tissue for light microscopy and immunoperoxidase staining was fixed in methyl Carnoy's solution [18] and embedded in paraffin. Four micrometer sections were stained with the periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin. The percentage of glomeruli exhibiting focal or global glomerulosclerosis was determined. Glomerulosclerosis was evaluated by an observer, who was unaware of the origin of the slides, and was evidenced by segmental increases in glomerular matrix, segmental collapse and obliteration of capillary lumina and accumulation of hyaline, and frequently was associated with synechial attachments to Bowman's capsule. Tubulointerstitial injury was defined as inflammatory cell infiltrates, tubular dilatation and/or atrophy, or interstitial fibrosis. Injury was graded according to Shih, Hines and Neilson [19] on a scale of 0 to 4 (0 = normal; 0.5 = small focal areas of damage; 1 = involvement of less than 10% of the cortex; 2 = involvement of 10 to 25% of the cortex; 3 = involvement of 25 to 75% of the cortex; 4 = extensive damage involving more than 75% of the cortex).

To analyze planar glomerular areas, the outer edges of all glomerular tufts (range 105 to 140) of each kidney were traced manually on a videoscreen and the encircled areas were determined by computerized morphometry, the technical details of which have been described elsewhere [20].

The additional 11 rats were fixed by *in vivo* total body perfusion with a buffered 1.5% glutaraldehyde solution as described previously [21]. Pieces of renal cortex were embedded in Epon 812 by standard procedures. Ultrathin sections were studied in a Philips Electron Microscope 301 to assess glomerular morphology.

The same 11 kidneys were also used to obtain semithin sections. These sections were stained with Azur II/Methylene Blue and were then used to evaluate early glomerular injury by assessing the frequency of pseudocysts. Seventy-five glomerular profiles in average were screened in each animal. The glomerular damage was scored by adding the percentage of glomerular profiles with one or two pseudocysts and percentage of glomerular profiles with three or more pseudocysts multiplied by two.

Immunoperoxidase staining

Four micrometer sections of methyl Carnoy's fixed biopsy tissue were processed by a direct or indirect immunoperoxidase technique as previously described [18]. Primary antibodies included:

- 1A4, a murine monoclonal antibody to an NH_2-terminal synthetic decapeptide of α-smooth muscle actin (gift of G. Gabbiani, Geneva, Switzerland) [22].
- D33, a murine monoclonal IgG_1 antibody against human muscle desmin (Dako, Glostrup, Denmark) [23].
- 19A2 (American Biotech Inc., Plantation, FL, USA), a murine IgM monoclonal antibody against human PCNA, which is expressed by actively proliferating cells. We have previously shown in angiotensin II infused rats, that cell proliferation as assessed by this anti-PCNA antibody correlates with the cell proliferation as assessed by the conventional method of ^{3}H-thymidine incorporation [9].
- ED1 (Bioproducts for Science, Indianapolis, IN, USA), a murine monoclonal IgG antibody to a cytoplasmic antigen present in monocytes, macrophages and dendritic cells [24].
- RP-3 (gift of F. Sendo, Yamagata, Japan), a murine monoclonal IgM antibody to rat neutrophils [25].
- OX-22 (Accurate Chemical Corporation, Westbury, NY, USA), a murine monoclonal IgG antibody to the high molecular weight form of the rat common leukocyte antigen expressed on B-lymphocytes and some T-lymphocytes [26].
- PL-1, a murine monoclonal antibody against rat platelets (gift of W.W. Bakker, Groningen, The Netherlands) [27].
- a polyclonal anti-rat fibrinogen/fibrin antibody (Cappel Laboratories, Cochranville, PA, USA).
- MPIIIB10 [1], a murine monoclonal antibody to rat osteopontin, MPI-IIB10 (obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, USA under contract NO1-HD-6-2915 from the NICHD) [28].
- an IgG fraction of polyclonal guinea pig anti-rat type I collagen [29] (provided by L. Iruela-Arispe, Seattle, WA, USA).
- affinity-purified polyclonal goat anti-human/bovine type IV collagen (Southern Biotechnology, Birmingham, AL, USA).
- an IgG fraction of polyclonal rabbit anti-rat laminin (Chemicon, Temecula, CA, USA).
- an affinity-purified IgG fraction of polyclonal rabbit anti-rat fibronectin (Chemicon).

For all biopsies, negative controls consisted of substitution of the primary antibody with equivalent concentrations of an irrelevant murine monoclonal antibody or normal rabbit or goat IgG. Evaluation of all slides was performed by an observer who was unaware of the origin of the slides.

To obtain mean numbers of proliferating cells or infiltrating leukocytes in glomeruli, more than 30 consecutive cross sections of glomeruli containing more than 20 discrete capillary segments were evaluated and mean values per kidney were calculated. To obtain total counts of proliferating cells or infiltrating leukocytes in the renal cortex or medulla over 40 grid fields (range 40 to 60), measuring 0.36 mm^2 each, were analyzed and, again, mean counts

Table 1. Basic characteristics of normotensive Milan rats or age-matched Wistar rats

Age *months*	Glomerulosclerosis *% of glom.*	Tubulointerstitial injury index	Right kidney weight *g*	Urinary protein excretion *mg/24 h*	Serum creatinine *mg%*
Milan					
2	0 ± 0	0 ± 0	1.0 ± 0.0	21 ± 4	0.3 ± 0.1
3	0 ± 0	0 ± 0	1.1 ± 0.1	42 ± 9	0.3 ± 0.1
4	2 ± 2	0 ± 0	1.2 ± 0.1	102 ± 14	0.3 ± 0.1
5	8 ± 3	1.0 ± 0.7	1.4 ± 0.1	167 ± 47	0.3 ± 0.0
6	8 ± 7	1.0 ± 0.7	1.2 ± 0.3	242 ± 94	0.5 ± 0.1
7	27 ± 10[a]	2.0 ± 0.7[a]	1.4 ± 0.2	438 ± 165[a]	0.4 ± 0.1
8	37 ± 12[a]	2.5 ± 0.4[a]	1.2 ± 0.1	415 ± 136[a]	0.5 ± 0.1
10	51 ± 12[a]	3.4 ± 0.6[a]	1.5 ± 0.3[a]	611 ± 160[a]	0.6 ± 0.1[a]
Wistar					
2	0 ± 0	0 ± 0	1.1 ± 0.2	4 ± 3	0.3 ± 0.1
10	4 ± 3	0.4 ± 0.5	1.4 ± 0.2	15 ± 8	0.4 ± 0.1

Glomerulosclerosis, tubulointerstitial injury index (**Methods**), serum creatinine and urinary protein excretion were determined in normotensive Milan rats ($N = 4$ each) or control Wistar rats ($N = 5$ each). Data are mean ± SD.

[a] $P < 0.05$ vs. 2-month-old rats

per kidney were obtained. For the evaluation of the immunoperoxidase stains for α-smooth muscle actin and desmin, each glomerular area or tubulointerstitial grid field was graded semiquantitatively, and the mean score per biopsy was calculated. Each score reflects mainly changes in the extent rather than intensity of staining and depended on the percentage of the glomerular tuft area or grid field showing positive staining: 0 = absent staining or less than 5% of the area stained, I = 5 to 25%, II = 25 to 50%, III = 50 to 75%, IV = >75%. We have recently described that this semiquantitative scoring system is not only reproducible among different observers but that the data also are highly correlated with those obtained by computerized morphometry [13].

In the case of immunostaining for α-smooth muscle actin and desmin, the periglomerular areas were assessed separately. Semiquantitative staining scores in these cases depended on the percentage of the interstitium immediately contiguous to Bowman's capsule showing positive staining: 0 = 0 to 5% stained, I = 5 to 25%, II = 25 to 50%, III = 50 to 75%, IV = >75%.

Immuno-electronmicroscopy

Kidney cortex of Milan normotensive rats perfusion fixed with 2% paraformaldehyde was used. Small pieces were immersed with 25% PVP-10,000 in 2.3 M sucrose and shock-frozen in liquid nitrogen. Sections of 100 nm thickness were cut in a Leica ultracryomicrotome at a temperature of −100°C. Sections were stained with monoclonal anti-desmin antibody (clone D33, Sigma) at a 1:10 dilution. A goat anti-mouse immunogold conjugate (IgG, 15 nm) was used as the second antibody.

Miscellaneous measurements

Urinary protein was measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, München, Germany) and bovine serum albumin (Sigma Chemical Corp., St. Louis, MO, USA) as a standard. Creatinine was measured using an autoanalyzer (Beckman Instruments GmbH, München, Germany).

Statistical analysis

All values are expressed as mean ± SD. Statistical significance (defined as $P < 0.05$) was evaluated using one way analysis of variance with modified *t*-tests performed using the Bonferroni correction.

Results

Progressive glomerulosclerosis, tubulointerstitial injury, proteinuria, and renal insufficiency develop in Milan rats past month 4

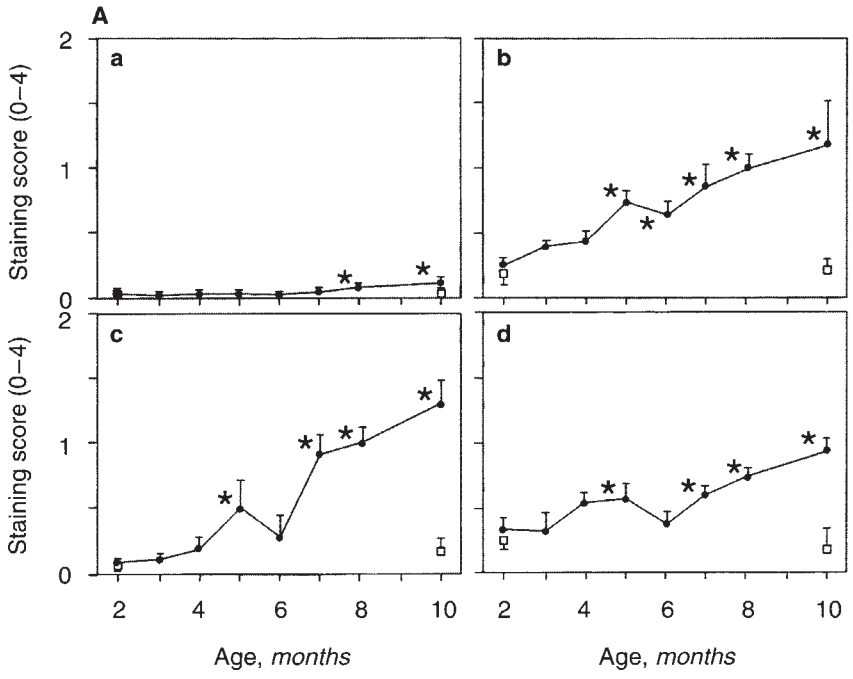
Mean systolic blood pressure was normal in 2-month-old Milan rats and did not change significantly during the study period (2 months old, 127 ± 5 mm Hg; 10 months old, 140 ± 14 mm Hg).

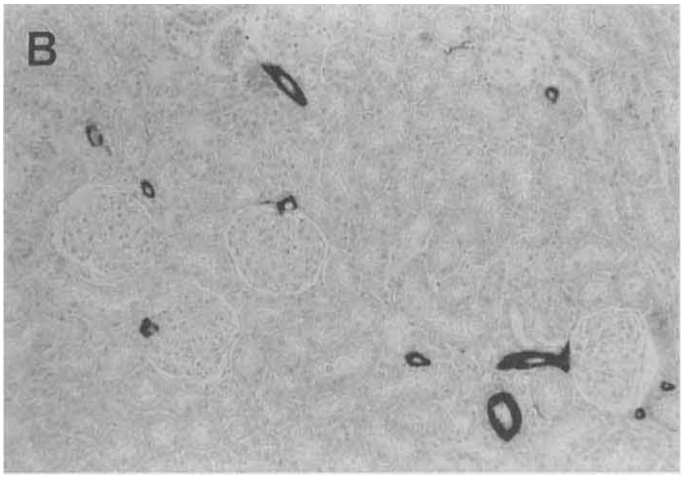
First evidence of focal segmental glomerulosclerosis was noted in the Milan rats at 4 to 5 months of age (Table 1). Glomerulosclerosis thereafter was progressive and at 10 months there was extensive segmental or global glomerulosclerosis (Table 1). Similarly, in the tubulointerstitium of Milan rats, first changes were noted at five months of age and then progressed towards widespread interstitial inflammation and damage (Table 1). Renal weight increased slowly with age (Table 1). The development of significant proteinuria was closely correlated with the above changes (Table 1). Serum creatinine values did not change until month 5, but progressively increased thereafter (Table 1).

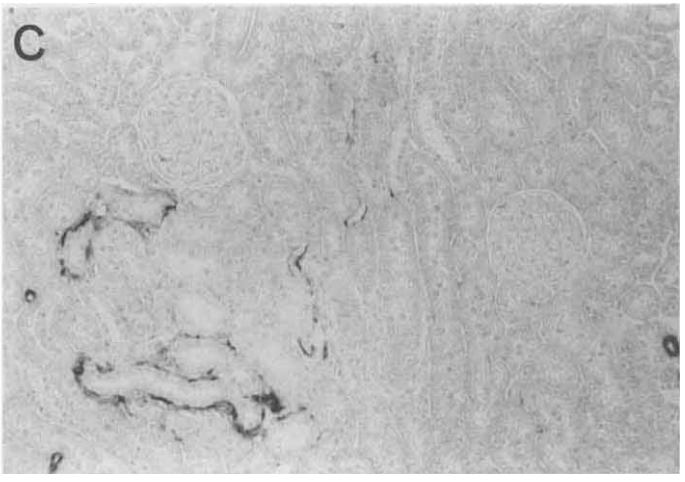
In Wistar control rats, no or only minor differences were noted between two-month-old and 10-month-old rats with respect to the prevalence of glomerulosclerosis, tubulointerstitial injury, and serum creatinine (Table 1). Both renal weight and proteinuria were increased in 10-month-old Wistar rats, as compared to two-month-old Wistar rats, albeit to a lesser degree than in Milan rats (Table 1).

Intermediate filament proteins: Progressive accumulation of cells positive for α-smooth muscle actin in the interstitium and desmin in the interstitium and glomeruli of Milan rats

Expression of α-smooth muscle actin in glomeruli of Milan rats was absent or minimal at all ages and only a minor, albeit significant, increase was detectable in 8- and 10-month-old rats (Fig. 1). In all other locations examined (interstitium adjacent to the glomeruli, cortex and medulla), the expression of α-smooth muscle actin was confined to vascular smooth muscle cells at two months of age and then progressively increased with age in interstitial cells (Fig. 1).







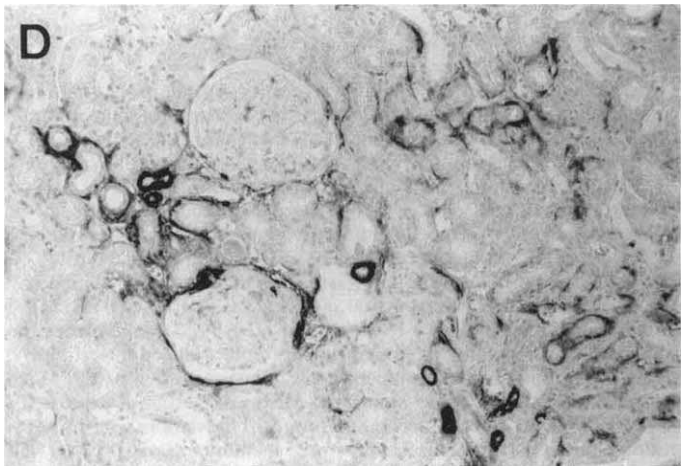
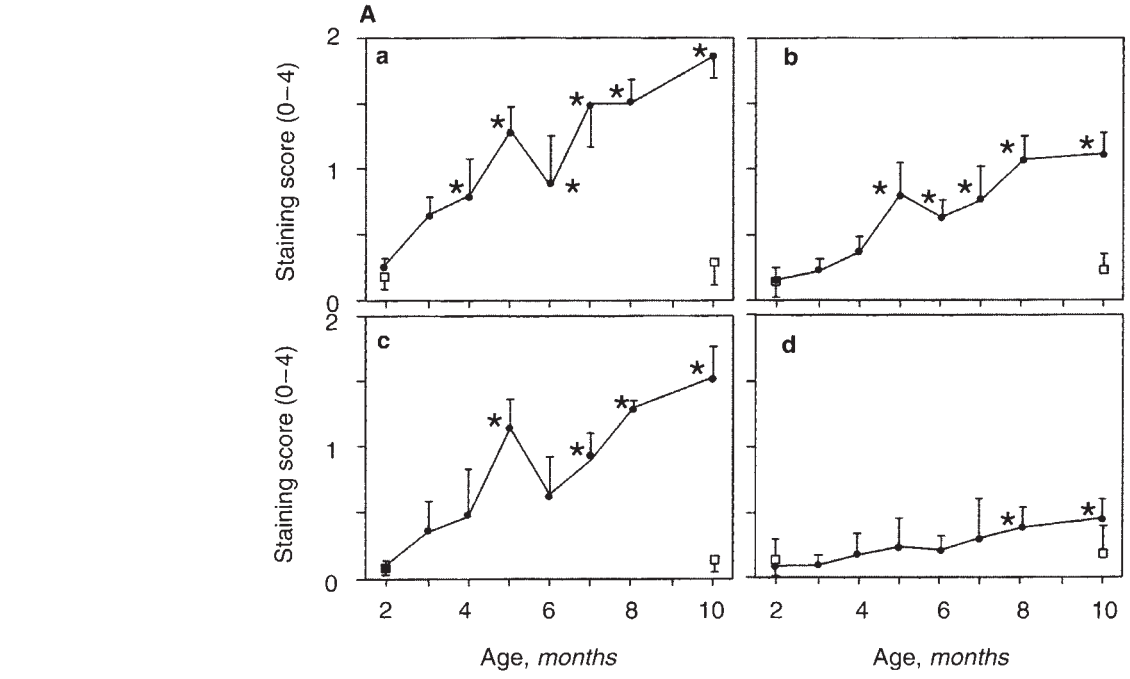
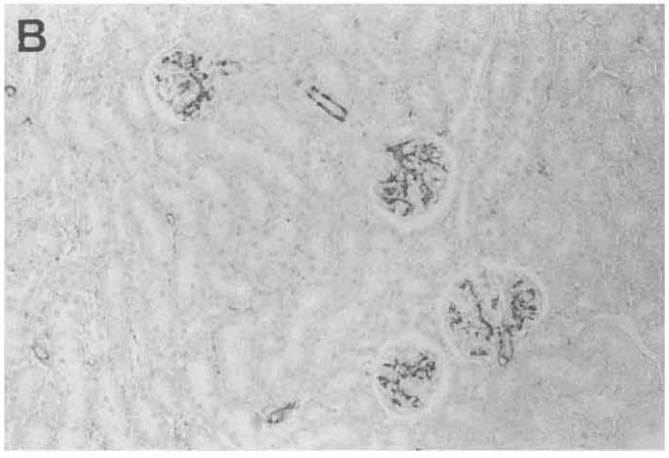
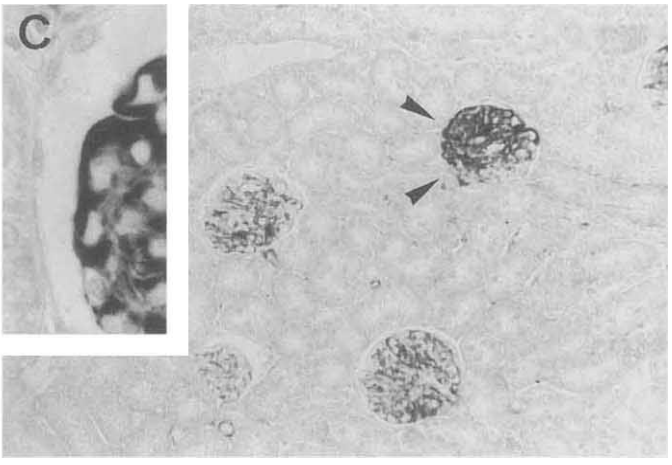


Fig. 1. *α-Smooth muscle actin.* (**A**) Immunostaining scores for α-smooth muscle actin in the following compartments: (a) glomeruli; (b) cortical interstitium; (c) glomerular capsule; (d) medullary interstitium. Data are mean ± SD, $N = 4$ each. $^*P < 0.05$ versus month 2. Symbols are: (●) MNS rats; (□) Wistar rats. (**B**) Renal α-smooth muscle actin immunostaining in a two-month-old MNS rat. α-Smooth muscle actin expression is confined to the vascular walls (magnification ×200). (**C**) Renal α-smooth muscle actin immunostaining in a four-month-old MNS rat. α-Smooth muscle actin expression is present in vascular walls and is focally expressed in the interstitium (×200). (**D**) Renal α-smooth muscle actin immunostaining in a 10-month-old MNS rat. α-Smooth muscle actin expression is markedly up-regulated in the widened interstitium but not in the glomeruli (×200).







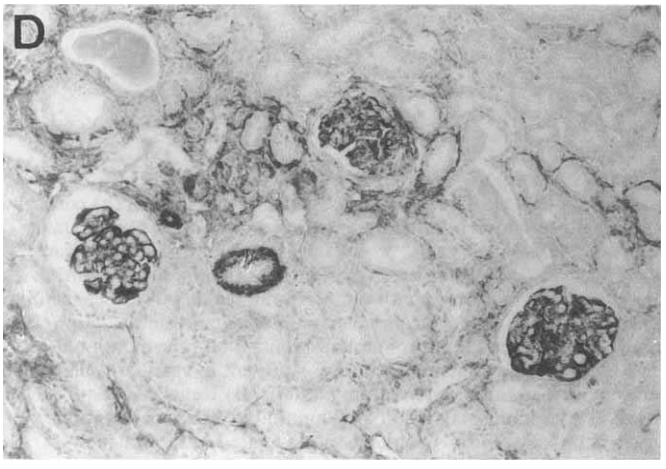


Fig. 2. *Desmin.* (**A**) Immunostaining scores for desmin in the following compartments: (a) glomeruli; (b) cortical interstitium; (c) glomerular capsule; (d) medullary interstitium. Data are mean ± SD, N = 4 each. $^*P < 0.05$ versus month 2. Symbols are: (●) MNS rats; (□) Wistar rats. (**B**) Renal desmin immunostaining in a two-month-old MNS rat. Desmin expression is confined to the mesangium and vascular walls (magnification ×200). (**C**) Renal desmin immunostaining in a three-month-old MNS rat. Desmin expression is focally up-regulated in glomeruli (arrows) (×200). The **insert** shows that the up-regulation is due to increased desmin expression in podocytes (×1000). (**D**) Renal desmin immunostaining in a 10-month-old MNS rat. Desmin expression is markedly up-regulated in glomeruli and in the widened interstitium (×200). (**E**) Immuno-electronmicroscopical demonstration of glomerular desmin in a four-month-old MNS rat. Desmin is expressed in primary processes of podocytes (arrows). Abbreviations are: E, endothelium; GBM, glomerular basement membrane (×36,000).

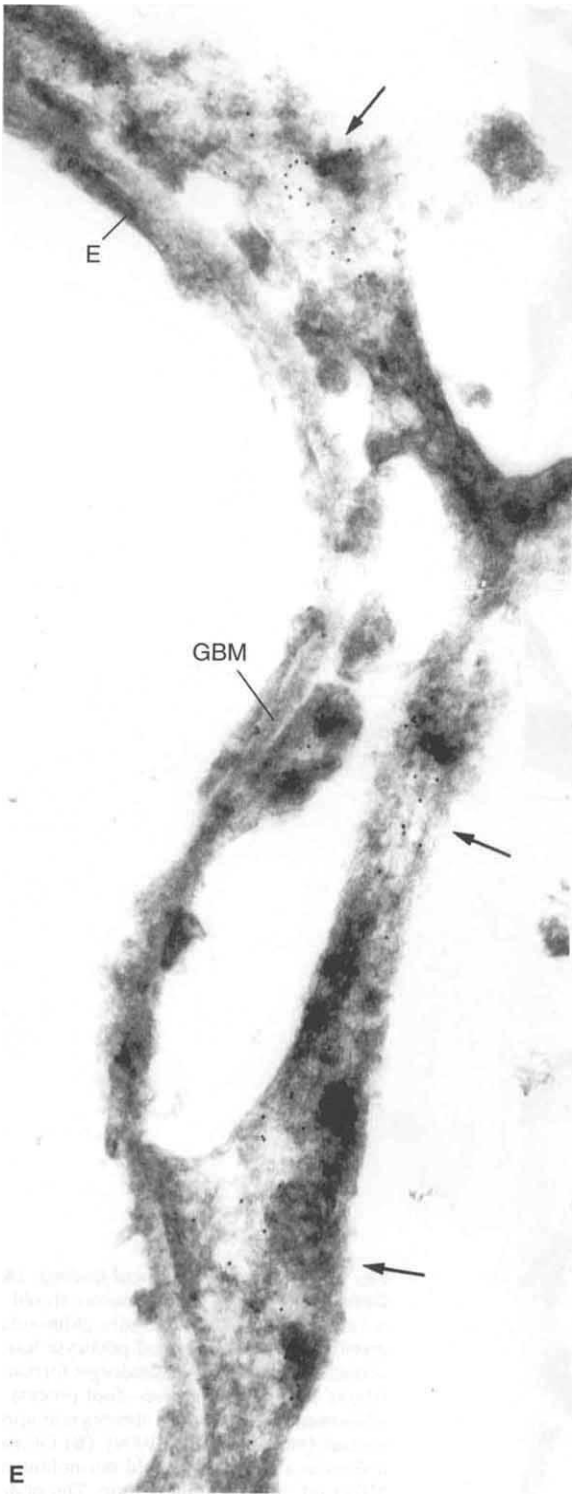


Fig. 2. *Continued.*

In two-month-old Milan rats desmin was expressed in the mesangium, vascular smooth muscle cells, as well as rare podocytes (Fig. 2B). Starting at three months of age, glomeruli focally exhibited markedly increased desmin (Fig. 2 A, C). Both by light microscopy (Fig. 2 C, D) and by immunoelectron microscopy (Fig. 2E) the increased expression of desmin appeared to be exclusively due to increased staining of podocytes. Within these glomeruli podocyte expression of desmin was variable at younger ages (Fig. 2C) and more uniform with increasing age (Fig. 2D). In the interstitium desmin expression was also up-regulated with increasing age (Fig. 2).

Kidneys of two-month-old control Wistar rats exhibited a staining pattern for α-smooth muscle actin and desmin that did not differ from that of two-month-old Milan rats. In contrast to Milan rats, no age-dependent changes were observed in the Wistar rats (Figs. 1A and 2A).

First evidence of podocyte injury is present in 3-month-old Milan rats

By electron microscopy, evidence of podocyte damage was noted in a focal pattern in occasional glomeruli of three-month-old MNS rats (Fig. 3A). These changes consisted of pseudocyst formation, foot process effacement and occasional local areas of denudation of the glomerular basement membrane (Fig. 3 A, B). No changes of the glomerular mesangium, endothelium or glomerular basement membrane were noted (Fig. 3 A, B). Evidence of podocyte damage became more widespread with increasing age of the rats, terminating in loss of podocytes in sclerotic glomeruli, a finding which was occasionally encountered as early as age of four months. Also, calculation of the glomerular damage score, a reflection of the frequency of pseudocysts, revealed a significant difference between rats aged 10 to 12 weeks and rats aged 14 to 19 weeks (80 ± 33, $N = 5$ vs. 124 ± 17, $N = 6$; $P < 0.05$).

Glomerular hypertrophy develops after month 2 in injured glomeruli

Analysis of glomerular cross sectional areas in sections stained with desmin antibody demonstrated a progressive increase of glomerular areas in MNS rats between two and five months of age (Fig. 4 and Table 2). When glomeruli were subdivided into those exhibiting a normal immunostaining pattern for desmin and those exhibiting augmented staining, it became apparent that the progressive increase of glomerular area with increasing age was exclusively due to the 10 to 20% larger size of glomeruli with an abnormal desmin staining pattern (Fig. 4 and Table 2).

The increase in glomerular areas with age as well as the area differences between glomeruli with normal and increased desmin expression (Fig. 4 and Table 2) were all statistically significant ($P < 0.01$) when calculated on the basis of individual area measurements but not when calculated on the basis of mean values per animal.

Proliferation: Tubulointerstitial cell proliferation, but not glomerular cell proliferation, progressively increases in Milan rats

Cell proliferation in all three compartments evaluated (glomeruli, cortex and medulla) was high in two-month-old Milan rats. At this age, cell proliferation per tissue area was most prominent in the renal cortex, where the majority of proliferating cells localized to tubules in a diffuse pattern. After month 2, the increased cell proliferation subsided and remained low in the glomeruli throughout the observation period (Fig. 5). In contrast, in both the renal cortex and medulla, cell proliferation increased again towards the end of the observation period (Fig. 5). At month 10,

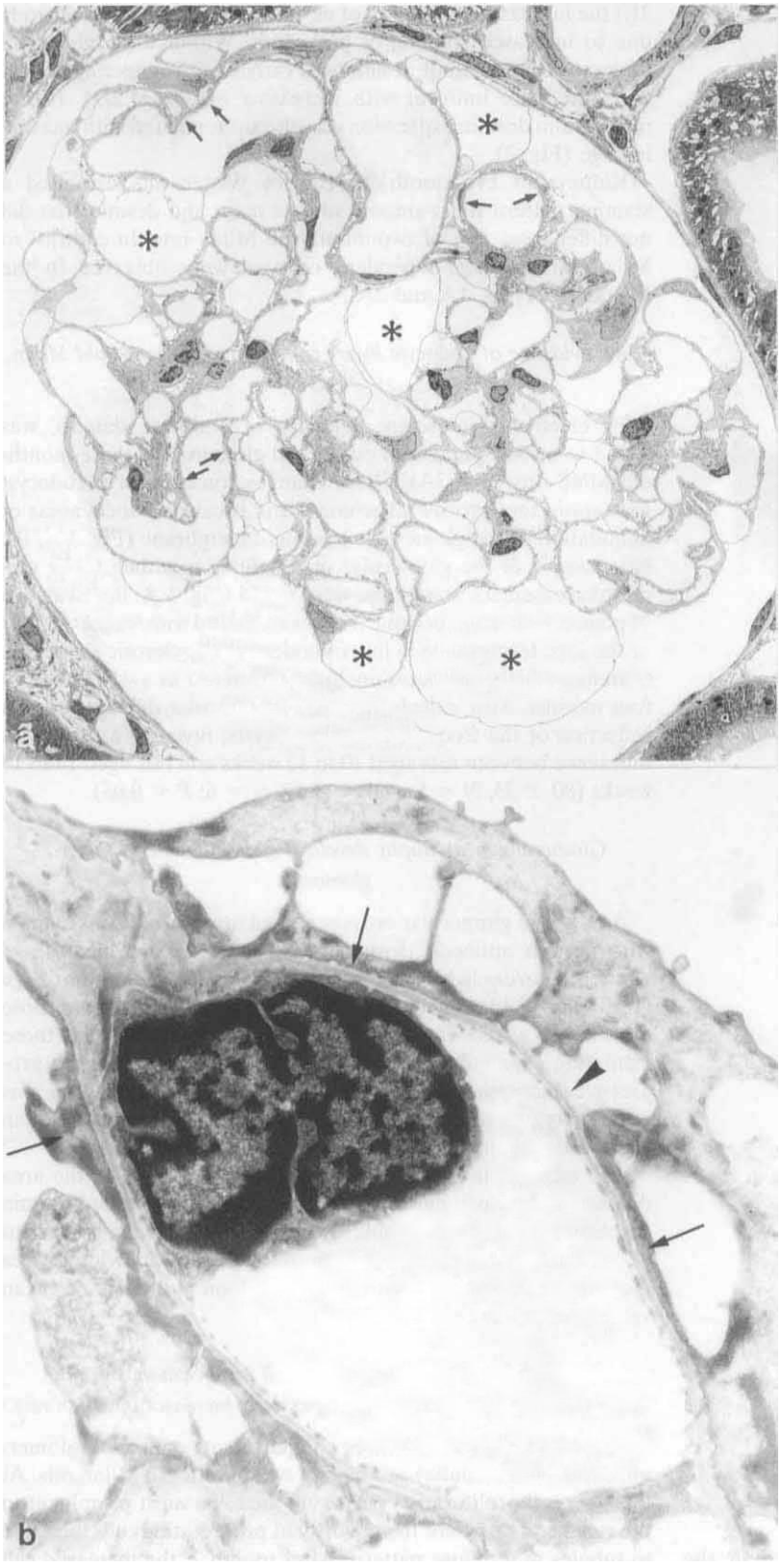


Fig. 3. *Electron microscopical findings.* (**A**) Glomerular lesions in a three-month-old normotensive Milan rat. Entire glomerular profile showing widespread podocyte lesions consisting of extensive pseudocyst formation (stars) and—less extensive—foot process effacement (arrows). The mesangium appears normal (magnification ×1000). (**B**) Glomerular lesions in a three-month-old normotensive Milan rat. Single capillary loop. The podocyte cover exhibits foot process effacement (arrows) as well as a local detachment from the glomerular basement membrane (arrowhead). The endothelium and the basement membrane are of normal appearance (×9000).

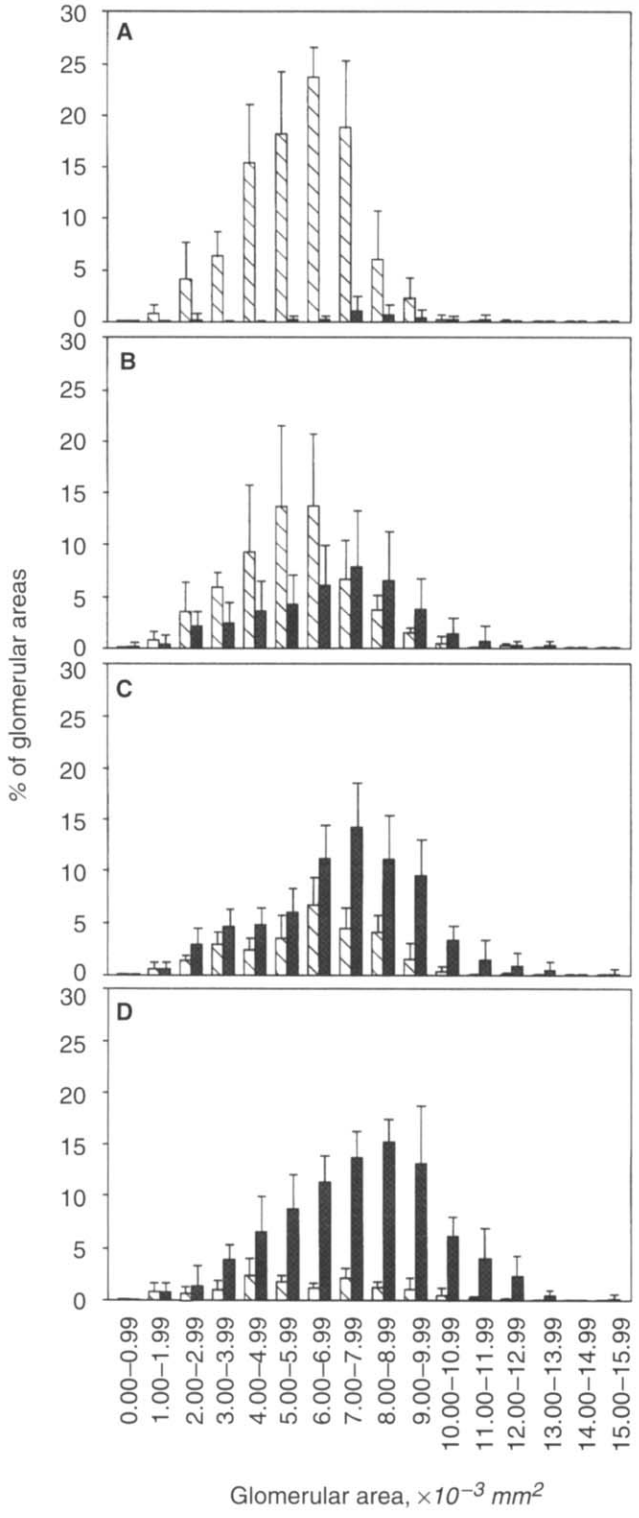


Fig. 4. *Glomerular hypertrophy.* Frequency distribution of glomerular cross sectional areas in kidneys from two- to five-month-old MNS rats (as assessed in renal sections stained with antibody to desmin and counterstained with methyl green). Glomerular cross sections are divided into those exhibiting normal desmin immunostaining (▨, staining scores 0 to 1; see **Methods**) or increased immunostaining (■, staining scores >1). Data are mean ± SD, N = 4 each. **A.** Month 2. **B.** Month 3. **C.** Month 4. **D.** Month 5.

Table 2. Glomerular areas of normotensive Milan rats

Age *months*	Mean area of all glomeruli $\times 10^{-3}$ mm^2	Mean area of glomeruli with normal desmin expression $\times 10^{-3}$ mm^2	Mean area of glomeruli with increased desmin expression $\times 10^{-3}$ mm^2
2	5.77 ± 1.64	5.69 ± 1.58	7.77 ± 1.91
3	6.47 ± 2.15	5.85 ± 1.90	7.03 ± 2.22
4	6.95 ± 2.28	6.26 ± 1.97	7.22 ± 2.34
5	7.44 ± 2.46	6.12 ± 2.53	7.63 ± 2.39

Mean glomerular tuft areas in MNS rats aged 2–5 months. Glomerular areas are also divided into those exhibiting normal desmin immunostaining (staining scores 0–1; see **Methods**) or increased immunostaining (staining scores >1). Data are mean ± SD, $N = 4$ each.

cell proliferation was increased focally, in particular in areas of leukocytic infiltration near the corticomedullary junction.

In Wistar control rats, cell proliferation in all three compartments was increased at two months of age in a similar range as that observed in young Milan rats (Fig. 5). At 10 months of age, glomerular counts of PCNA positive cells in Wistar rats were comparable to the Milan rats, but were lower in the cortex and medulla than in Milan rats (Fig. 5).

Inflammatory cells: Progressive tubulointerstitial infiltration of monocytes/macrophages in parallel to glomerular and tubular overexpression of osteopontin

Infiltration of glomeruli and the tubulointerstitium of Milan rats by monocytes/macrophages progressively increased after six months of age (Fig. 6A). Infiltration in the tubulointerstitium was focal with dense infiltrates in some areas and relatively normal appearing tissue in other areas. Prior to the monocyte/macrophage influx at five months of age, overexpression of osteopontin was noted in some cortical tubuli, particularly in areas of tubular dilation, which became more prominent with increasing age (Fig. 6 C, D). In the medulla, most tubules stained positively Milan rats at two months of age and no significant changes occurred with increasing age. In glomeruli no osteopontin expression was noted within the tuft, but capsular cells of damaged glomeruli frequently stained positive (Table 3).

No influx of neutrophils was noted in the kidneys of aging Milan rats. Staining for OX-22 positive lymphocytes showed a progressive influx after month 4 in the renal cortex but not in glomeruli or the renal medulla (Fig. 6B).

Staining for platelets revealed occasional clusters in glomerular capillaries of Milan rats over the age of five months, while no significant age-related changes were noted in the tubulointerstitium (Table 3). Staining for fibrinogen/fibrin also increased progressively with age in the glomeruli (Fig. 7 and Table 3), while there were no clear cut age-related changes in the tubulointerstitium.

None of the above age-related changes were noted in control two-month and 10-month-old Wistar rats (Figs. 6 A, B and Fig. 7).

Matrix: Progressive interstitial accumulation of various extracellular matrix proteins with increasing age of Milan rats

Staining for the extracellular matrix proteins type I collagen, type IV collagen, laminin, and fibronectin in two-month-old Milan

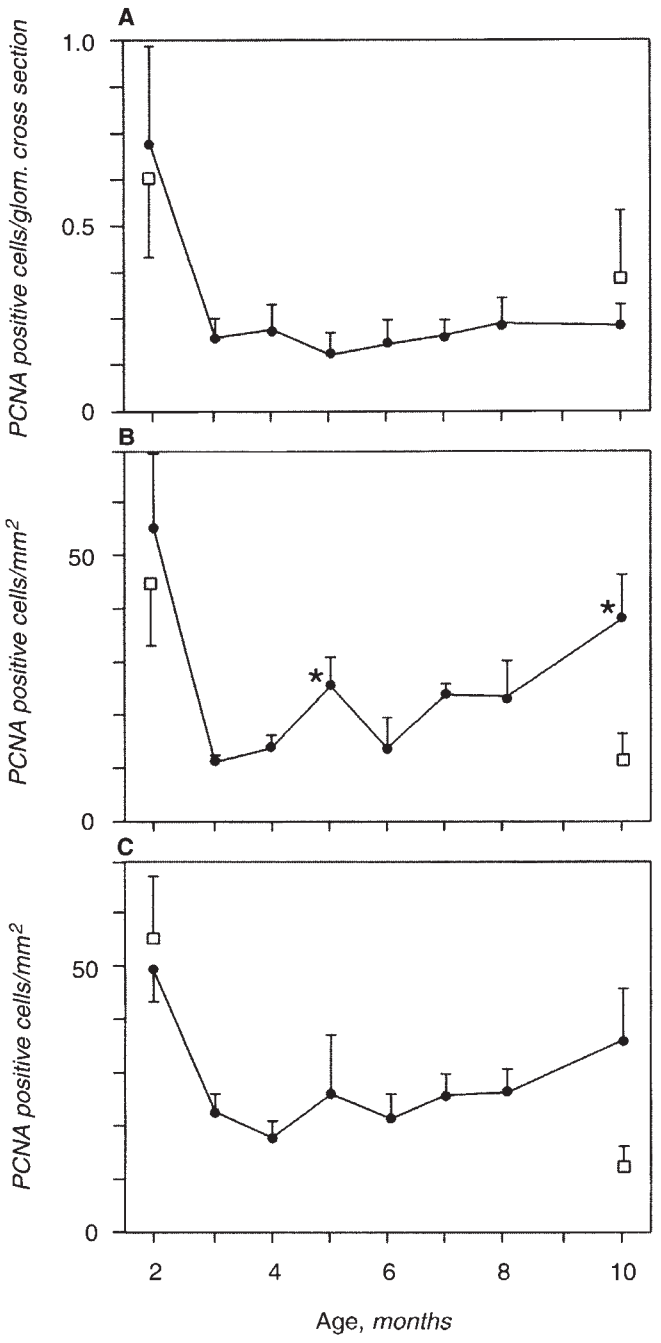


Fig. 5. *Cell proliferation.* PCNA positive cell counts were performed in the following compartments: (**A**) glomeruli; (**B**) cortical tubulointerstitium; (**C**) medullary tubulointerstitium. Data are mean ± SD, $N = 4$ each. *$P < 0.05$ versus month 2. Symbols are: (●) MNS rats; (□) Wistar rats.

and normal Wistar rats revealed patterns similar to those described previously in Wistar and Sprague-Dawley rat strains [9, 14, 30].

In Milan rats staining for all of the above matrix proteins markedly increased with age in the widened renal interstitium, in particular the cortical interstitium, and to a lesser degree also in damaged glomeruli (Fig. 8 and Table 3). First changes were noted after four months of age. Immunostaining for type I collagen was remarkable in that it increased in the interstitium, while glomerular staining for this collagen type was absent or minor even in 10-month-old rats with pronounced glomerulosclerosis (Fig. 8B).

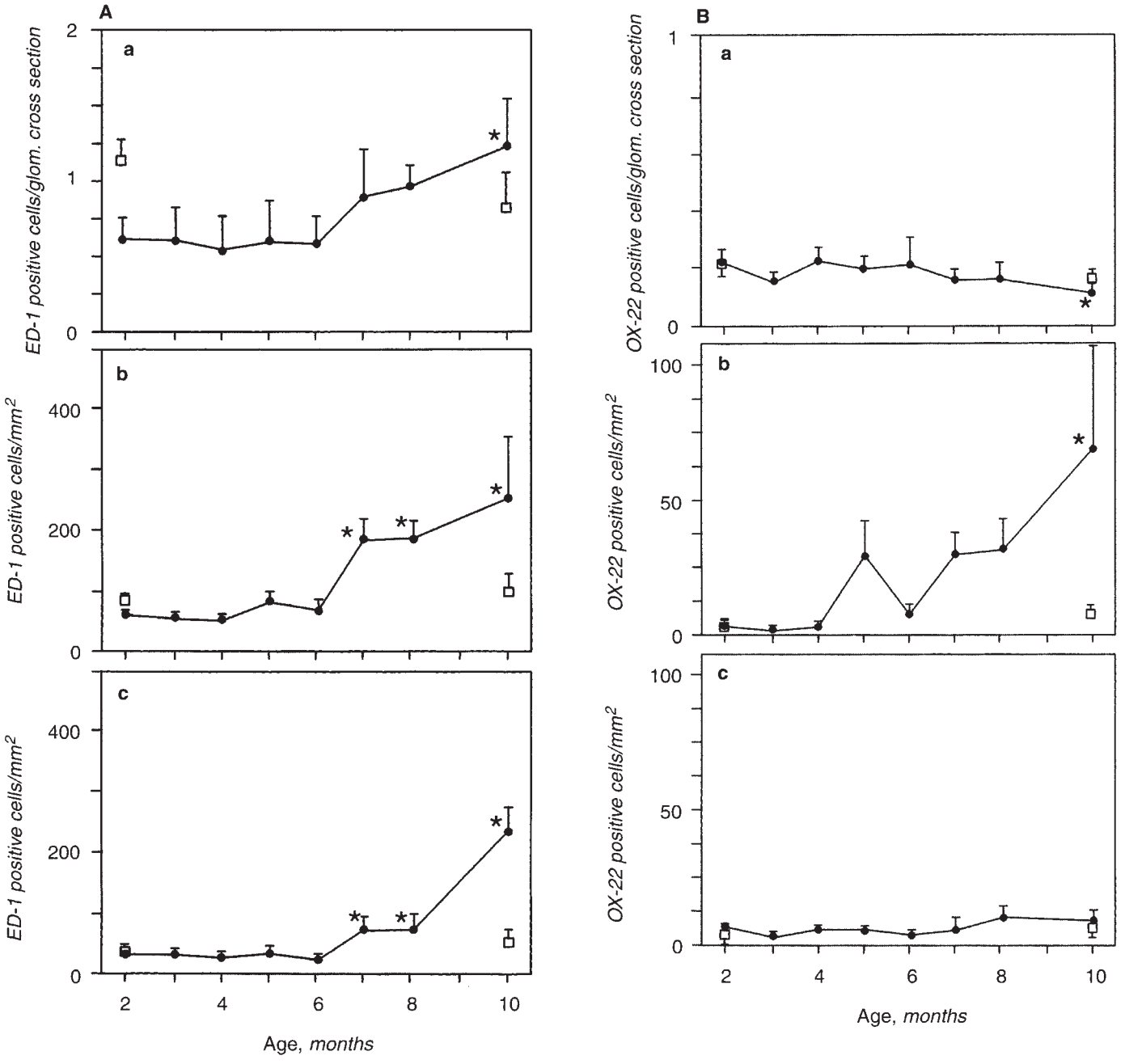
No age-related changes of extracellular matrix protein immunostaining were noted in Wistar control rats.

Discussion

Spontaneous, age-related development of glomerulosclerosis and interstitial renal fibrosis are well recognized events in various rat strains, and to a lesser extent also in humans [31–33]. MNS rats are remarkable in that glomerulosclerosis and interstitial fibrosis not only develop at a relatively young age but also in the absence of various known risk factors for progressive renal disease, such as systemic hypertension, hyperglycemia, immunological injury, preceding inflammatory events, glomerular hyperfiltration and apparently also independently of angiotensin II [16, 17, 34, 35]. Altered glomerular prostanoid production, in particular overproduction of thromboxane A_2, as well as hypercholesterolemia have been detected in MNS rats after the onset of proteinuria [36–38], and may therefore contribute to the progression but not the onset of renal disease. Indeed, measures that reduced glomerular thromboxane A2 overproduction and/or hypercholesterinemia afforded either no or only partial protection from glomerulosclerosis in these rats [37, 38]. Glomerular hypertrophy, another known risk factor for progressive renal disease [2], developed in MNS rats between the age of two and three months (Table 3). Prevention of glomerular hypertrophy by a low protein diet effectively retarded the development of glomerulosclerosis in these rats [17]. Preliminary findings show that glomerular hypertrophy in MNS rats appears to result from increased glomerular protein synthesis rather from decreased proteolysis (L. Fels and S. Kastner, personal communication). Therefore, it may be speculated that an as yet undefined dysregulation of glomerular protein synthesis contributes to the development of glomerulosclerosis in this model.

The most remarkable finding of the present study was the very early up-regulation of desmin expression in the glomeruli of young Milan rats, which preceded almost all other glomerular changes (Table 3). The desmin overexpression localized to podocytes. Desmin has been identified as a non-specific, yet sensitive marker of podocyte injury [39]. In agreement with data of a previous study [40], the very early onset of podocyte injury was

Fig. 6. *Inflammatory cell influx and chemoattractant proteins.* (**A**) Monocyte/macrophage counts in the following compartments: (**a**) glomeruli; (**b**) cortical tubulointerstitium; (**c**) medullary tubulointerstitium. Data are mean ± SD, N = 4 each. *$P < 0.05$ versus month 2. Symbols are: (●) MNS rats; (□) Wistar rats. (**B**) Counts of OX-22 positive lymphocytes in the various compartments [(**a**) glomeruli; (**b**) cortical tubulointerstitium; (**c**) medullary tubulointerstitium]. Data are mean ± SD, $N = 4$ each. *$P < 0.05$ versus month 2. Symbols are: (●) MNS rats; (□) Wistar rats. (**C**) Renal osteopontin immunostaining in a two-month-old MNS rat. Osteopontin is weakly expressed in some cortical tubules (×200). (**D**) Renal osteopontin immunostaining in a 10-month-old MNS rat. Osteopontin expression is up-regulated in some tubular segments (×200).


A
a
b
c
ED-1 positive cells/glom. cross section
ED-1 positive cells/mm2
0
1
2
0
200
400
2
4
6
8
10
Age, months
B
a
b
c
OX-22 positive cells/glom. cross section
OX-22 positive cells/mm2
0
1
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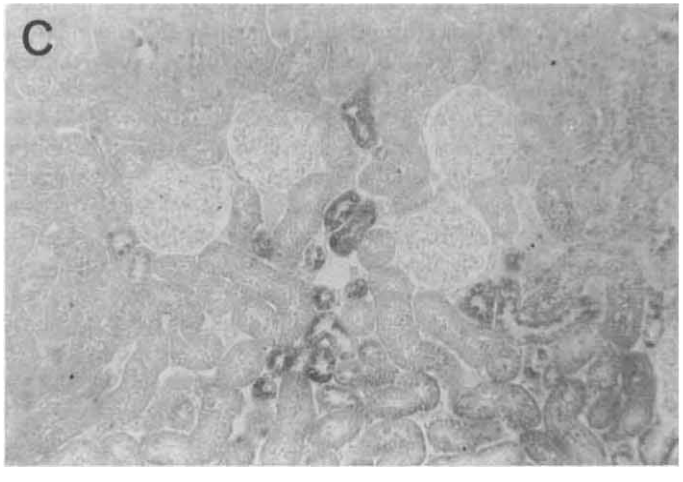
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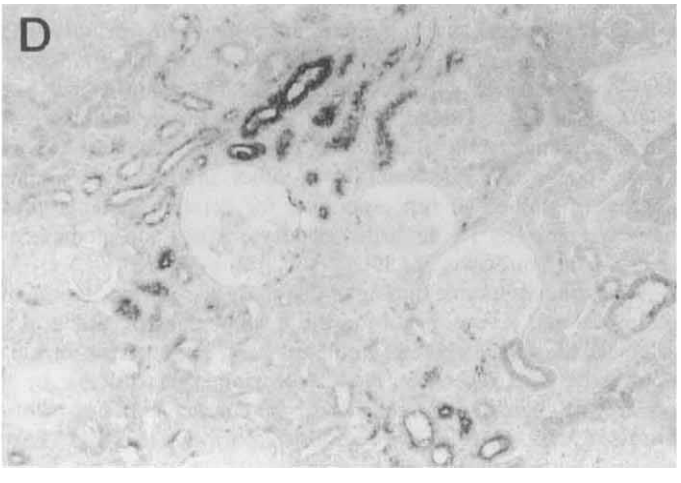
D

Table 3. Semiquantitative summary of age-related changes in the kidneys of normotensive Milan rats

	Age *months*	3	4	5	6	7	8	10
Proteinuria		→	↑	↑	↑	↑	↑	↑
Serum creatinine		→	→	→	↑	→	↑	↑
Glomerulosclerosis		→	→	↑	↑	↑	↑	↑
Tubulointerstitial injury		→	→	↑	↑	↑	↑	↑
α-Smooth muscle actin expression	glomerular	→	→	→	→	→	→	↑
	tubuloint.[a]	→	↑	↑	↑	↑	↑	↑
Desmin expression	glomerular	↑	↑	↑	↑	↑	↑	↑
	tubuloint.[a]	→	↑	↑	↑	↑	↑	↑
Planar area	glomerular	↑	↑	↑	n.d.	n.d.	n.d.	n.d.
Pseudocysts	glomerular	↑	↑	↑	n.d.	n.d.	n.d.	n.d.
Proliferation	glomerular	[b]	→	→	→	→	→	→
	tubuloint.[a]	[b]	→	↑	→	↑	↑	↑
Monocytes/macrophages	glomerular	→	→	→	→	↑	↑	↑
	tubuloint.[a]	→	→	→	→	↑	↑	↑
Osteopontin expression	glomerular	→	→	→	→[c]	→[c]	→[c]	→[c]
	tubuloint.[a]	→	→	↑	↑	↑	↑	↑
OX-22 positive lymphocytes	glomerular	→	→	→	→	→	→	↓
	tubuloint.[a]	→	→	↑	→	↑	↑	↑
Neutrophils	glomerular	→	→	→	→	→	→	→
	tubuloint.[a]	→	→	→	→	→	→	→
Platelets	glomerular	→	→	→	↑	↑	↑	↑
	tubuloint.[a]	→	→	→	→	→	→	→
Fibrinogen/fibrin	glomerular	→	→	↑	→	↑	↑	↑
	tubuloint.[a]	→	→	→	→	→	→	→
Matrix protein accumulation[d]	glomerular	→	→	↑	↑	↑	↑	↑
	tubuloint.[a]	→	→	↑	↑	↑	↑	↑

Symbols are: →, ↑, no change or increase, respectively, compared to 2-month-old Milan rats n.d. is not determined.
[a] Only renal cortical changes are shown
[b] Due to the persistent renal growth in 2 months old rats, all changes are expressed as changes compared to 3 months old rats
[c] Only parietal glomerular epithelial cells but no cells within the tuft showed increased expression of osteopontin at these time points
[d] Glomerular staining for type I collagen did not increase with age

also confirmed by electron microscopy, which showed widespread podocyte pseudocyst formation and foot process effacement (Fig. 3). On a functional basis, podocyte damage was evidenced by the early onset of proteinuria, as it is believed that podocytes represent a central part of the glomerular barrier to plasma proteins [41]. Since podocyte damage preceeded almost all other renal changes in MNS rats (Table 3), our findings support the theory that podocyte damage with subsequent GBM denudations and tuft adhesions at the Bowman's capsule are crucial steps in the pathogenesis of segmental glomerulosclerosis [42–45]. Our data also suggest that the only other very early abnormality in MNS rats (Table 3), namely, that glomerular hypertrophy (including the increased glomerular protein synthesis) occurs either independently of or secondary to podocyte damage, but is not the cause of podocyte damage. Hence, glomerular hypertrophy in the MNS rats was relatively mild and unlikely to result in mechanical strain of the podocytes. Furthermore, in other models with similar degrees of glomerular tuft expansion, for example mesangioproliferative anti-Thy 1.1 nephritis, podocyte injury and glomerulosclerosis do not occur [30, 39].

A second remarkable finding of this study was the low degree of mesangial cell activation during the development of glomerulosclerosis. Mesangial cell activation has been evidenced in various progressive and reversible models of glomerular disease by a phenotypic change of the cells to exhibit characteristics of myofibroblasts, that is, cells involved in scar formation [46]. These characteristics include:

(1) De novo α-smooth muscle actin expression. The paucity of this actin isoform in the glomeruli of aging Milan rats indicates that its expression depends on the presence of other factors, such as systemic hypertension, increased angiotensin-II levels, and/or mesangiolysis [6, 9, 47].

(2) Increased cell proliferation. This feature was prominent in very young MNS rats but also in young Wistar control rats, and therefore likely relates to the physiological renal maturation process. After month 2, the increased glomerular cell proliferation ceased completely in MNS rats. In contrast, both after 5/6 nephrectomy in rats as well as in growth hormone transgenic mice, increased glomerular cell proliferation accompanies glomerulosclerosis [6, 48]. The findings of the present study do not corroborate those of Pugliese et al [49], who demonstrated *in vitro* that mesangial cells derived from eight-month-old MNS rats exhibited a significantly higher spontaneous proliferation rate than those of one-month-old animals.

(3) *De novo expression of glomerular type I collagen.* This was also rare in aging MNS rats despite the accumulation of other matrix proteins in the sclerotic glomeruli. This differential accumulation of matrix proteins is unusual in progressive renal disease, since in many other cases all examined types of matrix proteins accumulated in sclerotic lesions [12, 14, 30, 50, 51]. However, in aging Fisher 344 rats glomerular overexpression of type IV collagen, laminin and fibronectin, but not of types I or III collagen, has been noted [52].

Despite the relatively unique features of the MNS rats in the

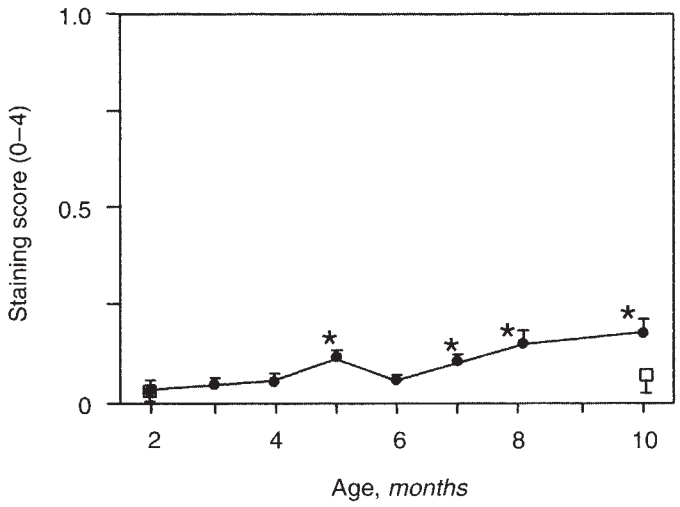

Fig. 7. *Fibrinogen/fibrin.* Immunostaining scores for fibrinogen/fibrin in glomeruli. Data are mean ± SD, $N = 4$ each. *$P < 0.05$ versus month 2. Symbols are: (●) MNS rats; (□) Wistar rats.

early stages of renal disease, the pathological changes during the later development of progressive renal disease (summarized in Table 3) show remarkable similarities with those of other experimental models. Thus, in MNS rats, angiotensin II infused rats, rats with Goldblatt hypertension (two-kidneys-one-clip), as well as in 5/6 nephrectomized rats an early overexpression of the cytoskeletal proteins desmin and α-smooth muscle actin, that is, markers of myofibroblasts [53], has been noted in the tubulointerstitium [9, 13, 14]. Similarly, in human renal disease, interstitial fibroblasts also aquire the ability to express α-smooth muscle actin [54]. Later stages of the various disease models are characterized by mild glomerular platelet and fibrinogen accumulation [6] and a prominent glomerular and interstitial leukocyte, in particular monocyte/macrophage, influx [6, 9, 14], which may be driven in part by the overexpression of chemotactic factors such as osteopontin in the damaged renal tissue [55]. Leukocyte infiltration paralleled accumulation of multiple extracellular matrix proteins both in glomeruli and the interstitium as well as the development of progressive glomerulosclerosis, interstitial fibrosis and proteinuria [12].

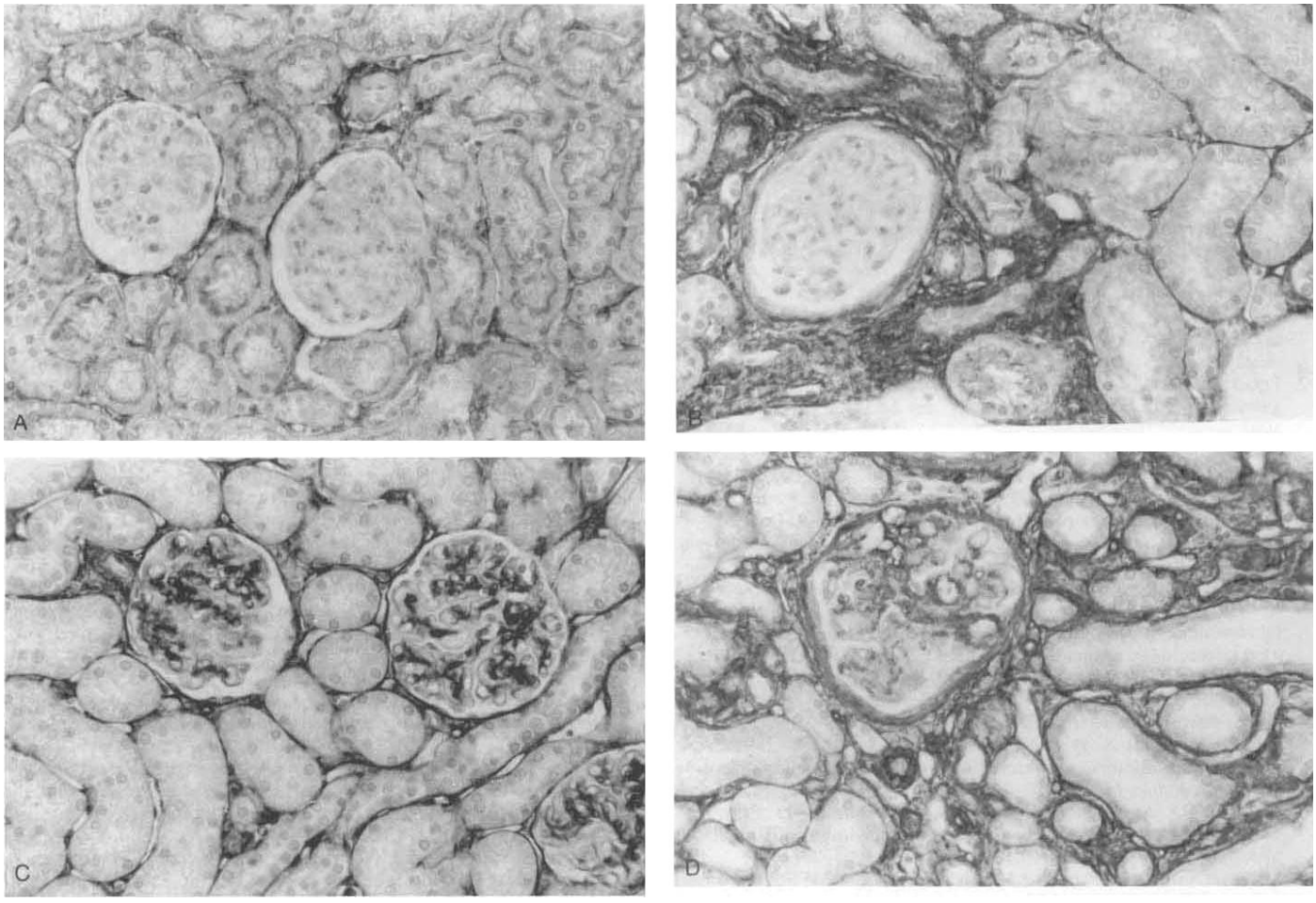

Fig. 8. *Extracellular matrix proteins.* (**A**) Renal immunostaining for type I collagen in a two-month-old MNS rat. Type I collagen is expressed in the tubulointerstitium but not in glomeruli (magnification ×400). (**B**) Renal immunostaining for type I collagen in a 10-month-old MNS rat. Type I collagen staining is up-regulated in the widened interstitium but not in glomeruli (×400). (**C**) Renal immunostaining for type IV collagen in a two-month-old MNS rat. Type IV collagen is expressed in the glomeruli and in the tubulointerstitium (×400). (**D**) Renal immunostaining for type IV collagen in a 10-month-old MNS rat. Type IV collagen staining is up-regulated in sclerotic glomeruli and in the widened interstitium (×400).

In summary, our findings are noteworthy for the paucity of evidence to suggest mesangial cell activation and, in particular, the acquisition of myofibroblast features by these cells. Rather, our data support the hypothesis that in the case of the MNS rats podocyte damage, exerted by a yet unknown mechanism, plays a central role in the pathogenesis of glomerulosclerosis. The data also fit the theory that podocyte damage and subsequent proteinuria may be involved in the onset of secondary tubulointerstitial disease, in particular fibroblast activation. In these respects MNS rats show marked similarities to the aminonucleoside nephrosis model of toxic podocyte injury, in which heavy proteinuria is also associated with progressive tubulointerstitial disease [8, 56]. Later stages in the development of glomerulosclerosis and interstitial fibrosis in MNS rats exhibit many immunohistochemical features which resemble those of various other models of progressive renal disease, and therefore support to the existence of a final common pathway of renal injury. MNS rats thus exhibit many features of human primary focal and segmental glomerulosclerosis and represent a useful model to study both the pathogenesis and therapeutic interventions in this glomerular disorder.

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