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A new single nephron model of focal and segmental glomerulosclerosis in the Munich-Wistar rat

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A new single nephron model of focal and segmental glomerulosclerosis in the Munich-Wistar rat. The hypothesis that damage to the visceral epithelial cell plays a central role in the pathogenesis of focal and segmental glomerulosclerosis was tested by injecting saponin solutions of increasing concentration (0.1, 0.3, 0.6 and 1.0 mg/ml) in Bowman's space of superficial glomeruli in the Munich-Wistar rat. The microinjections were performed both with and without intermittent clamping of the renal vessels during two minutes. After 8 to 14 days the injected glomeruli were examined by light microscopy. The injected glomeruli were classified as, normal (NL), showing visceral epithelial cell damage (VECD), showing focal and segmental glomerulosclerosis (FSGS) or showing global sclerosis (GS). Swelling and intracellular vacuolation of the visceral epithelial cells (VEC) were considered as VECD. FSGS-lesions were seen most frequently in the glomeruli injected with 10 nl of a saponin solution with a concentration higher than 0.3 mg/ml. In view of the light microscopic lesions four glomeruli in a 0 mg/ml, the 0.1 mg/ml and the 0.6 mg/ml saponin groups were examined after 40 minutes with transmission electron microscopy (TEM) to evaluate the selectivity of the lesions. In the 0 and 0.1 mg/ml group only occasional limited fusion of the foot processes of the podocytes was seen. In the 0.6 mg/ml group segmental lysis of the VEC without ultrastructural damage to the capillary basement membrane or the endothelial and mesangial cells was seen. It is concluded that it is possible to induce direct segmental lysis of the visceral epithelial cells in a single glomerulus, and that this damage to the visceral epithelial cells is related to the development of focal and segmental glomerulosclerosis.

The term focal and segmental glomerulosclerosis (FSGS) is used to denote a primary glomerular disease [1–3]. The same term is also used to describe a lesion seen in several other renal diseases [4]. In experimental animals such as the rat several conditions seem to provoke FSGS: aging [5], surgical reduction of the functional renal mass [6–10], administration of toxic substances such as puromycin aminonucleoside [11–13] and adriamycin [14–16].

Surgical reduction of the renal mass and the administration of adriamycin [17] or puromycin aminonucleoside [18] are additive in provoking FSGS. In all experimental models [19–26] as in human FSGS [27, 28] lesions of the visceral epithelial cell precede the formation of FSGS, so it is currently hypothesized that damage to the VEC plays a pivotal role in the pathogenesis of FSGS. To test this hypothesis we developed a new technique to directly damage VEC in a single glomerulus.

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Methods

Animals

Forty-four female Munich-Wistar (MWF/Ztm) (MW) rats weighing less than 165 g were used for these experiments. In contrast to male MW rats, female MW rats do not develop spontaneous proteinuria [29] and FSGS lesions when weighing less than 165 g [30]. Rats were fed standard rat chow and water *ad libitum*.

Experimental design

Micropuncture of Bowman's space of superficial glomeruli was performed and 10 nl of a saponin solution (SS) in 0.05% Lissamine green in saline was injected at a rate of 10 nl/min. We chose the cell detergent saponin because it has been shown that this agent can remove the endothelial cells of arterial wall fragments *in vitro*, without damaging the underlying basement membrane or smooth muscle cells [31–33]. Based on these reports and on our own preliminary experiments we used four different concentrations: 0.1 mg/ml, 0.3 mg/ml, 0.6 mg/ml and 1.0 mg/ml. The microinjections were made with and without clamping the renal artery and vein with a microclamp during two minutes. Biopsies of the injected glomeruli and of control areas in the same kidney were taken after 8 to 14 days and studied by light microscopy.

To study the early lesions, glomeruli injected with a saponin solution were selectively biopsied after 40 minutes to be studied by transmission electron microscopy. Four glomeruli were injected with a 0 mg/ml saponin solution to exclude mechanical damage caused by the technique. Based on the light microscopic results four glomeruli were injected with a 0.1 mg/ml saponin solution and another four with a 0.6 mg/ml saponin solution. Each time two glomeruli were injected with and two without clamping the renal vessels. Each glomerulus was punctured in a different animal to allow a very specific biopsy as described subsequently.

Table 1. Characteristics of the animals and results in the non-clamped group

Rat	Age *days*	Weight *gram*	Punctured glomeruli *N*	Saponin conc. *mg/ml*	Time of biopsy *days*	NF	NL	VECD	FSGS	GS
							N			
1	48	132	2	0.1	10		2			
2	50	145	3	0.1	10		3			
3	50	148	3	0.1	10		2		1	
4	51	147	2	0.1	10		2			
5	58	151	3	0.3	8		3			
6	51	148	7	0.6	14		1		1	5
7	46	114	5	1.0	10		2		1	2
8	49	122	3	1.0	8	3				
9	50	123	3	1.0	10		2	1		

Abbreviations are: NF, not found; NL, normal; VECD, visceral epithelial cell damage; FSGS, focal and segmental glomerulosclerosis; GS, global sclerosis; *N*, number.

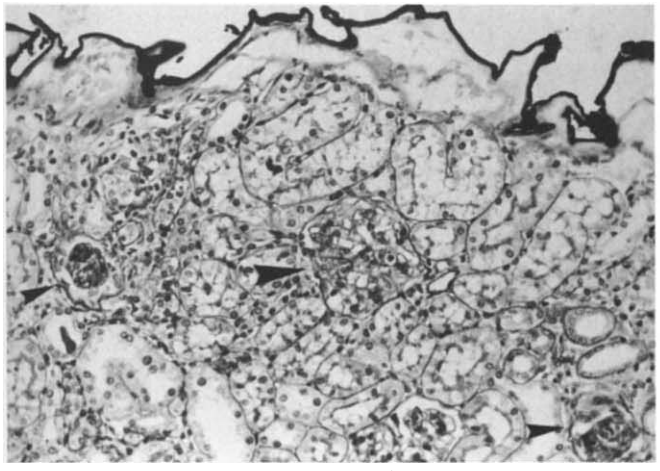

Fig. 1. *Three glomeruli close to an obliquely sectioned capsule marked with Chinese ink.* Two glomeruli have a global sclerosis (thin arrow), the middle glomerulus shows a segmental sclerosis (thick arrow). 0.6 mg/ml SS, without clamping the renal vessels (×100).

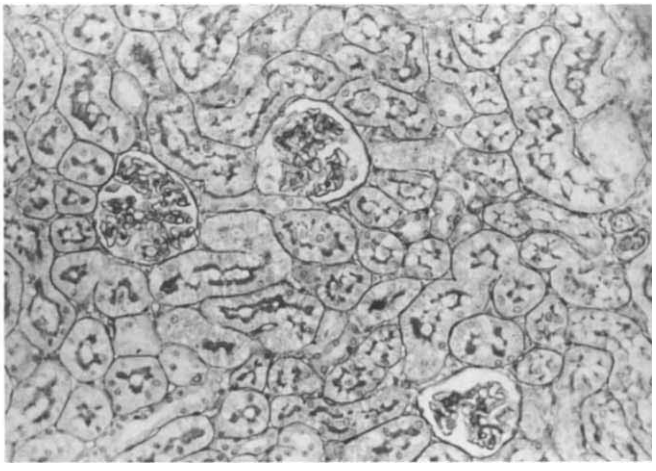

Fig. 2. *Three glomeruli in a triangular configuration, at distances of 1 glomerulus and 3 glomeruli from each other.* No lesions are present. 0.3 mg/ml SS without clamping the renal vessels (×100).

Micropuncture

Micropunctures were performed as classically described [34]. Briefly, the animals were anesthetized with Nembutal (Sanofi-Labaz NV, Brussels, Belgium) 40 to 60 mg/kg intraperitoneally and placed on a temperature controlled operation table (Klaus Effenberger, PFAFFING/Attel, Germany). During the whole procedure the temperature of the animal was monitored with a rectal thermometer (Klaus Effenberger) and kept at 37°C. A catheter was inserted in the left femoral vein and saline was infused at a rate of 2 ml/hr throughout the procedure. This catheter was also used to inject 0.05 ml of 5% Lissamine green (Lissamine Grün SF Chroma-gesellschaft, Schmid GMBH&Co., Stuttgart-Untertürckheim, Germany) in saline. Through a left subcostal incision the left kidney was gently freed from the surrounding fat, placed in a kidney cup, immobilized by partial embedding in Agar 3% and constantly irrigated with saline at 37°C. The micro pipette (pulled on a micro pipette puller by Klaus Effenberger) for the micropuncture had a classic beveled tip (ground on a motor-driven grinding machine by Klaus Effenberger) with an external diameter of 10 μm, and was filled with the saponin solution (Sigma Chemical Company, St. Louis, Missouri, USA). The pipette was mounted on a micro-infusion pump (Microperfusionspumpe 1-50 nl/min; Wolfgang Hampel, Neu-Isenburg, Germany) allowing a constant delivery of minute amounts of fluid. Mostly three to five superficial glomeruli in a certain configuration, with the glomeruli lying not more than a few hundred μm from each other, were injected. The glomeruli that were mechanically damaged (evidenced by a glomerular bleeding) were not used, and another area was chosen to inject glomeruli. Before and after the puncture the functional integrity of the glomeruli was tested by injecting 0.05 ml of a 5% Lissamine green solution in saline in the femoral vein. After this procedure the area of the punctured glomeruli was marked with Chinese ink and a drawing of the configuration of the glomeruli was made. The abdomen was closed in two layers and after ligation of the femoral vein the inguinal incision was sutured.

For TEM evaluation only one glomerulus in a kidney was

Table 2. Characteristics of the animals and results in the clamped group

Rat	Age *days*	Weight *gram*	Punctured glomeruli *N*	Saponin conc. *mg/ml*	Time of biopsy *days*	NF	NL	VECD	FSGS	GS
								N		
1	57	144	2	0.1	10			2		
2	48	132	2	0.1	10		2			
3	60	164	3	0.1	13	3				
4	50	145	3	0.1	10		3			
5	53	146	3	0.1	10		3			
6	56	162	3	0.1	10		3			
7	58	151	2	0.3	8	2				
8	60	154	3	0.3	10		1	2		
9	34	97	4	0.3	10		4			
10	41	126	3	0.3	10		3			
11	42	119	2	0.3	10		1		1	
12	43	125	2	0.3	14		1	1		
13	44	118	3	0.6	9		1		1	1
14	46	152	2	0.6	10					2
15	49	142	3	0.6	10				1	2
16	49	142	3	0.6	10		1		2	
17	50	151	3	0.6	10		1		1	1
18	31	116	2	1.0	10					2
19	46	114	7	1.0	10		2			5
20	49	122	3	1.0	8		1			2
21	49	123	3	1.0	9			1	1	1
22	50	123	4	1.0	10		4			
23	50	119	3	1.0	8			1	2	

Abbreviations are: NF, not found; NL, normal; VECD, visceral epithelial cell damage; FSGS, focal and segmental glomerulosclerosis; GS, global sclerosis; *N*, number.

punctured to allow a selective biopsy with a self-devised mini-biopsy needle. Also each time a control biopsy of a non-punctured zone was performed to be studied by LM.

Biopsies

After 8 to 14 days the animal was anaesthetized with Nembutal 40 mg/kg, the left kidney was placed in the kidney cup, immobilized by partial embedding in Agar 3% and irrigated with saline at 37°C. The kidney surface was inspected through the microscope to localize the configuration of the punctured glomeruli marked with the Chinese ink. After identification a biopsy of this zone was made immediately after removal of the kidney and the tissue was placed in Bouin's fluid. A control biopsy of a non-punctured zone of the same kidney was made in every experiment.

The biopsies for TEM were taken in the *in situ* kidney with a self-devised miniature version of a biopsy needle used for percutaneous kidney biopsies in humans. This technique yielded a conical biopsy of 2.5 mm length, with the maximal diameter (800 μm) at the surface of the kidney, tapering towards the deeper tissue. The superficial glomerulus localized in the widest part of the tissue core was the injected glomerulus.

Light microscopy

The biopsies were routinely processed through paraffin and sections of 3 μm were made parallel to the kidney surface. The tissue block was completely cut in numbered serial sections. Each third section was picked up and prepared for staining. By this method glomerular lesions of less than 10 μm would be present in at least one section. Eventually all serial sections in the marked zone were prepared [16]. Sectioning parallel to the renal surface allowed positive identification of the glomeruli in the spatial configuration noted *in vivo*. The sections were stained with a PAS method. Some glomeruli could not be found (NF). The positively identified glomeruli were either normal (NL) or showed visceral epithelial cell damage (VECD), focal and segmental glomerulosclerosis (FSGS), consisting of an adhesion with segmental increase of mesangial matrix, hyalinosis and collapse of capillaries, or global sclerosis (GS).

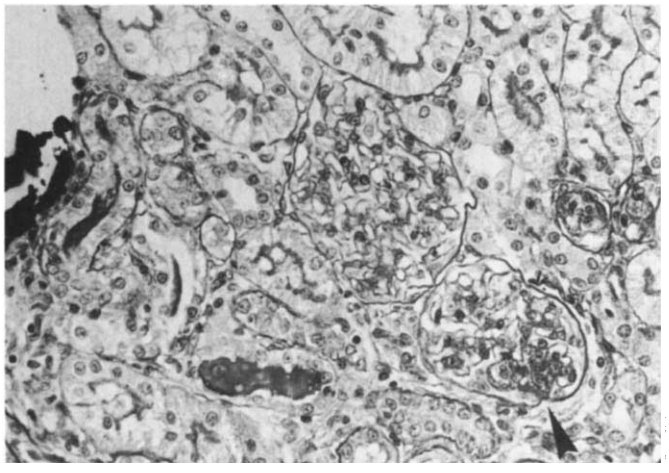

Fig. 3. *Two glomeruli close to an obliquely sectioned capsule marked with Chinese ink.* One glomerulus shows a segmental sclerosis (thick arrow). The other one is normal (and not injected). 0.6 mg/ml SS, while clamping the renal vessels (×160).

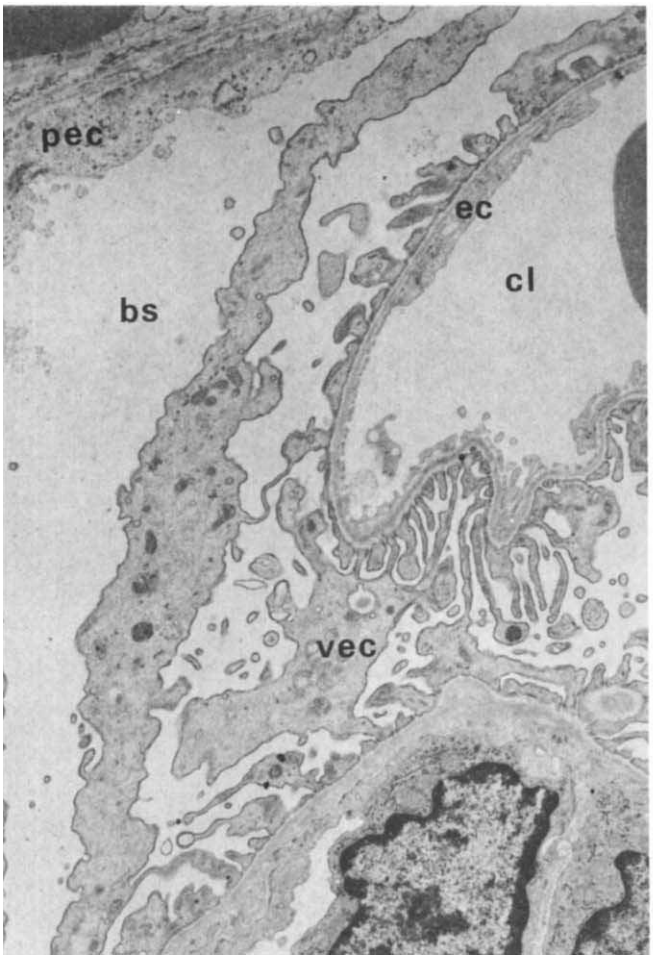

Fig. 4. *Electron micrograph from a glomerulus injected with a 0.1 mg/ml SS with clamping of the renal vessels.* Biopsy after 40 minutes (×7000). Abbreviations are: vec, visceral epithelial cell; cl, capillary lumen; pec, parietal epithelial cell; ec, endothelial cell; bs, Bowman's space. No lysis of vec.

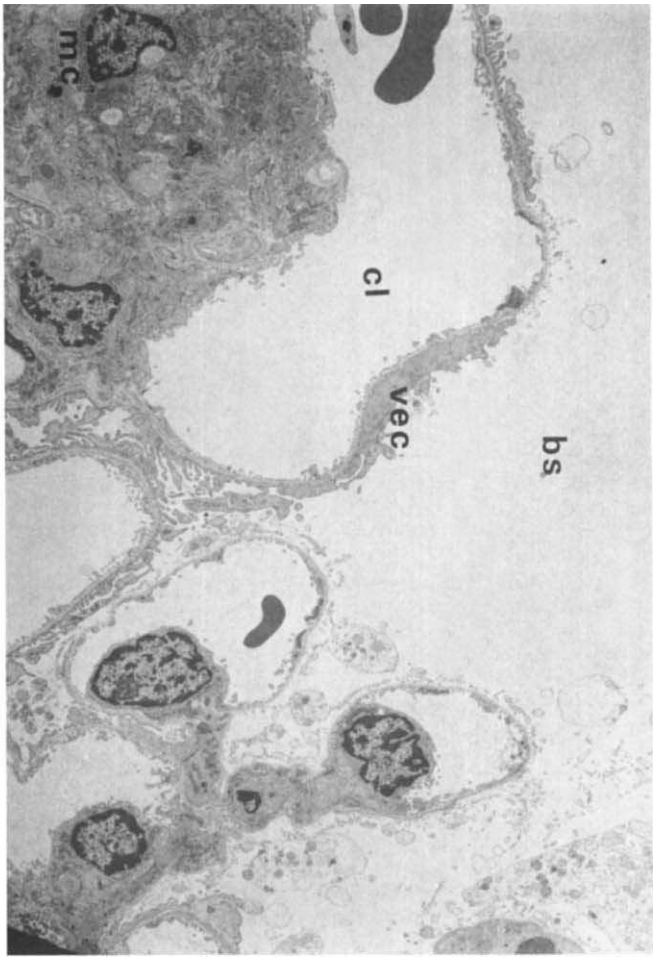

Fig. 5. *Electron micrograph from a glomerulus injected with a 0.6 mg/ml SS with clamping of the renal vessels.* Biopsy after 40 minutes (×2800). Abbreviations are: vec, visceral epithelial cell; cl, capillary lumen; mc, mesangial cell; bs, Bowman's space. Segmental lysis of vec. Segmental fusion of foot processes of vec. Intact mc.

Transmission electron microscopy

The biopsies were routinely processed after fixation in glutaraldehyde through progressive dehydration steps and embedded in epon. The kidney surface side was oriented towards the sectioning side of the epon-embedded biopsy and the glomerulus was first localized in semithin sections of 1.5 μm thickness. Ultrathin sections of 50 nm were made at several levels through the glomerulus and studied in a transmission electron microscope (Zeiss EM10).

Results

Light microscopy

Initial experiments were performed to define the saponin concentration that would lead to sclerotic lesions (FSGS and GS). Most sclerotic lesions appeared in the glomeruli injected with the 0.6 mg/ml saponin solution (Table 1, Figs. 1 and 2). Some glomeruli were seen to have a transient vasoconstriction during the micropuncture, while others remained normal. To avoid possible interference of a variable glomerular filtration rate in the subsequent experiments, the renal vessels were clamped for two minutes during the saponin injection of a glomerulus.

In these experiments (Table 2) FSGS lesions were predominantly seen in the 0.6 mg/ml group (Fig. 3). In the 1 mg/ml group mainly GS lesions were seen. In the glomeruli injected with lower saponin concentrations (0.1 or 0.3 mg/ml) most glomeruli were normal or showed only visceral epithelial cell damage. The observed FSGS lesions were not confined to the perihilar region of the tuft but had a random localization, the site of the microinjection in the glomerulus also being random.

Even with careful serial sectioning not all punctured glomeruli were identified. This was due to the fact that some tissue blocks were not cut strictly parallel to the kidney surface (in the area of the punctured glomeruli), so that the recorded configuration was not present in one section plane. This, however, implies that none of the punctured glomeruli had segmental lesions larger than 10 μm since these would have been identified in the sections examined.

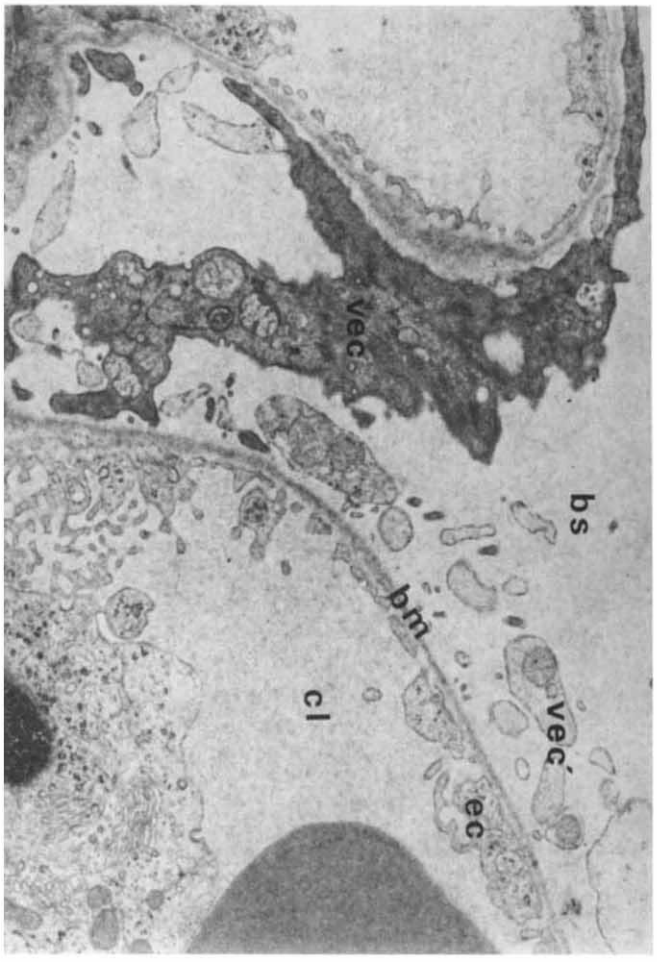

Fig. 6. *Electron micrograph of a glomerulus injected with a 0.6 mg/ml SS with clamping of the renal vessels.* Biopsy after 40 minutes (×14000). Abbreviations are: vec, visceral epithelial cell (with fusion of foot processes); vec', lysed visceral epithelial cell; cl, capillary lumen; bs, Bowman's space; bm, basement membrane. Lysis of vec. Fusion of foot processes. Intact basement membrane and endothelial cells.

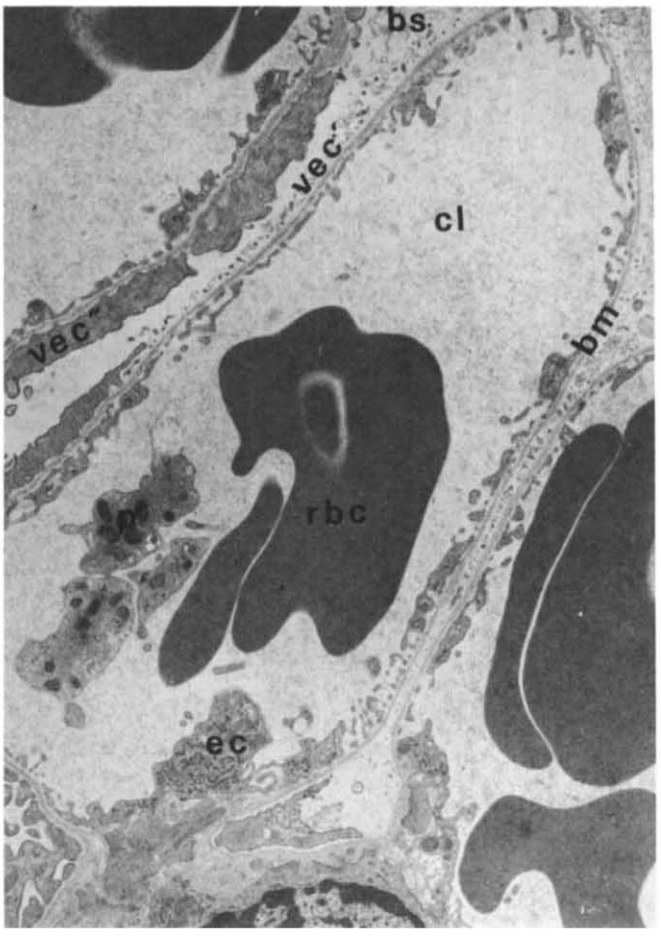

Fig. 7. *Electron micrograph of a glomerulus injected with a 0.6 mg/ml SS without clamping of the renal vessels.* Biopsy after 40 minutes (×7500). Abbreviations are: vec, visceral epithelial cell (intact); vec', lysed vec; vec'', vec with fusion of foot processes; cl, capillary lumen; bs, Bowman's space; ec, endothelial cell; rbc, red blood cell; p, platelet; bm, basement membrane. Lysis of vec and fusion of the foot processes in another vec. Intact basement membrane and endothelial cells.

Glomeruli from the non-punctured control areas were always normal.

Transmission electron microscopy

In the control group and the 0.1 mg/ml saponin group occasionally very limited fusion of the foot processes of the VEC was seen. All other structures were normal (Fig. 4).

In the 0.6 mg/ml saponin group segmental lysis of the VEC was observed. The basement membrane, the endothelial cells and the mesangial cells remained intact. Focal lysis of some parietal and some proximal tubular epithelial cells was noted. In one glomerulus of the clamped group and in one glomerulus of the nonclamped group aggregation of blood elements with formation of some fibrin fibers in a few capillaries was observed (Figs. 5 to 7). No differences were found between the endothelia of areas denuded of the visceral epithelium and non-denuded areas of the capillaries (Fig. 8), and no differences were found in the aspect of the endothelia between glomeruli injected with 0.6 mg/ml and those injected with 0.1 mg/ml. There were no qualitative differences between clamped and non-clamped glomeruli.

Discussion

Our data show that direct and severe damage to the visceral epithelial cells is related to the formation of focal and segmental glomerulosclerosis. In the first experiments (without clamping the renal vessels) the injection of the higher concentration of saponin (0.6 mg/ml or more) results in the occurrence of FSGS and GS. Some glomeruli had a transient vasoconstriction during the microinjection while others remained normal. This could lead to a variable glomerular filtration rate resulting in a variable saponin concentration in Bowman's space, and a variable contact time of the saponin solution with the podocytes. To avoid this problem we clamped the renal vessels during the microinjection in a second group of experiments. This was expected to lead to a more reproducible contact of saponin with the visceral epithelial cells. In this group we indeed saw a more constant occurrence of FSGS. The FSGS lesions were also

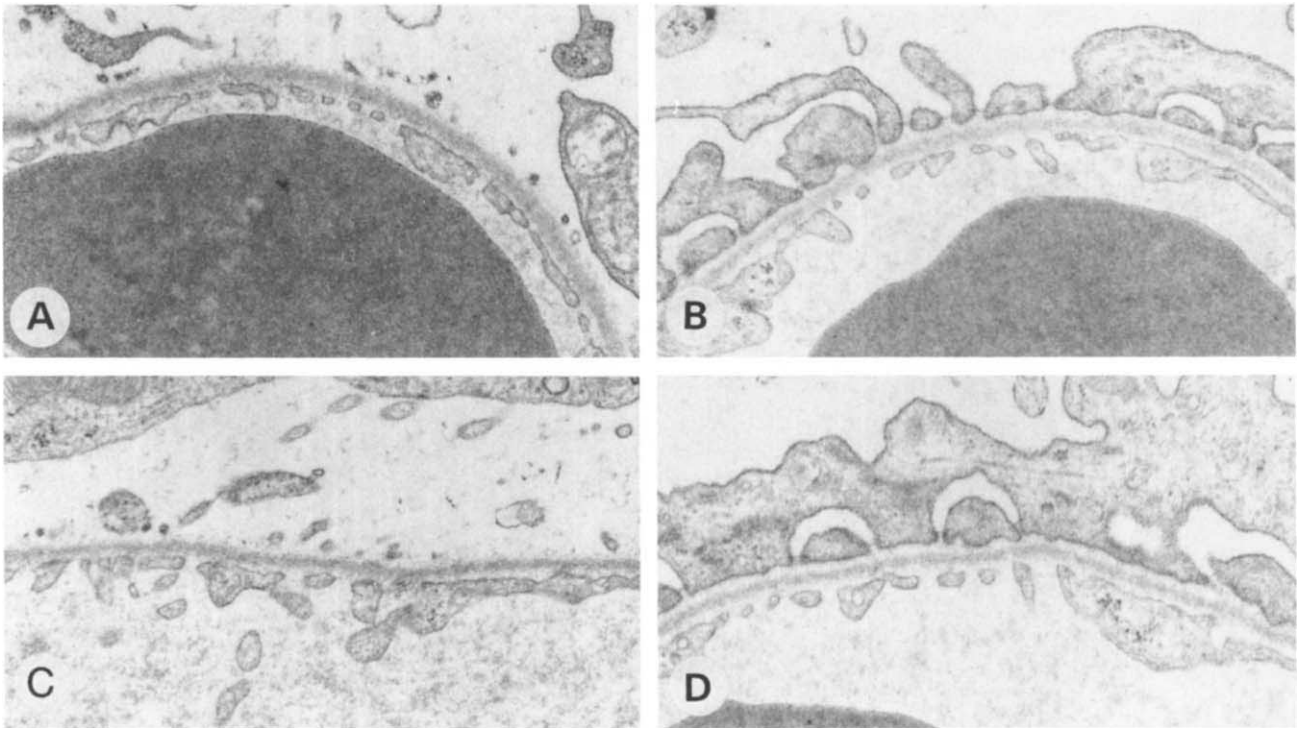

Fig. 8. *Electron micrograph of capillary walls of glomeruli injected with 0.6 mg/ml without clamping of the renal vessels.* Biopsy after 40 minutes (×7500). In panels **a** and **c** the basement membrane is denuded; in panel **b** and **d** the vec are still present. b and d are from the same capillary, a, b, c, are from different capillaries. All capillaries are from the same glomerulus. No differences in morphology of the endothelium can be seen.

randomly localized in the glomeruli as the localization of the microinjection was random in the glomerulus. In the toxic model of puromycin nephrosis in the rat, the focal sclerosis lesions were randomly dispersed, as the toxic effect to the podocytes was also random [35]. In the unilateral nephrectomy model in the rat, predominance of focal sclerosis at the vascular pole was noted [36], because in this model aneurysmal dilation of the glomerular capillaries occurs at the vascular pole, subjecting the podocytes to a maximal strain [38]. The short (2 min) interruption of the bloodstream did not produce lesions in the glomeruli not injected, nor in glomeruli injected with control solutions.

The TEM studies show that the early lesions of the capillary tuft are limited to segmental lysis of the VEC. It has been shown by others that the concentrations used in our studies did not damage the basement membrane in arterial walls, nor was the structure or the function of the smooth muscle cells altered with contact times up to 45 minutes (1 min in our experiments) [30–32] and with concentrations of saponin up to 1 mg/ml. Moreover, the glomeruli were observed to resume filtration at the end of the procedure, without bleeding. This observation would be unlikely if endothelium or basement membranes had been damaged. Finally, our TEM studies show that in the 0 mg/ml group, the 0.1 mg/ml group and the 0.6 mg/ml group no lesions of the basement membrane or the endothelial cells were detected. Parietal and tubular epithelial cells were also damaged as they have direct contact with the saponin solution. In contrast to the visceral epithelial cells, these have the capacity of regeneration, and no data are available for a primary role for the parietal epithelium in the pathogenesis of FSGS. Moreover, destruction of tubular epithelium by a variety of toxins does not lead to FSGS.

Although in some capillaries aggregation of blood elements and fibrin fibers were seen, no morphologically demonstrable damage to the endothelial cells could be detected.

Focal and segmental glomerulosclerosis is accompanied by the formation of an adhesion between the capillary tuft and Bowman's capsule. This implies that visceral epithelial cells are removed and that the capillary is covered by parietal epithelial cells [37]. Our findings demonstrate that loss of visceral epithelial cells leads to the formation of an adhesion. As the VEC is unable to divide [17, 38–41] replacement of the VEC over the denuded areas of the basement membrane is impossible. Detachment of visceral epithelial cells can be caused directly by epithelial cell strain as in glomerular hypertrophy or by epithelial cell injury by toxic components. This is in accordance with the hypothesis formulated by Fries et al [17], and elaborated by Rennke and Klein [4].

In conclusion, this study demonstrates that direct destruction of visceral epithelial cells in a single glomerulus is associated with the development of segmental glomerulosclerosis. Further studies with this model of glomerular damage may lead to a better analysis of the different steps in the pathogenesis of the experimental and clinical forms of focal and segmental glomerulosclerosis.

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