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# Proteinuria in rats induced by serum from patients with collapsing glomerulopathy

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**Proteinuria in rats induced by serum from patients with collapsing glomerulopathy.**

*Background.* Primary collapsing glomerulopathy recurs posttransplant, raising the possibility of circulating factors implicated in the pathogenesis of the disease.

*Methods.* To determine the presence of circulating factors in collapsing glomerulopathy patients, we tested serum from those patients in an in vivo assay. Eleven groups of rats received serum from collapsing glomerulopathy patients, idiopathic focal segmental glomerulosclerosis (FSGS) or healthy subjects in its native form, isolated IgG, or serum without IgG. The presence of proteinuria and creatinine clearance were determined. Histopathologic analysis included light, immunofluorescence, and electron microscopy.

*Results.* Collapsing glomerulopathy rats developed proteinuria while rats injected with serum from FSGS and healthy subjects did not. Rats injected with serum of collapsing glomerulopathy in its native form developed marked proteinuria ($99.2 \pm 42$ mg/24 hours at day 5, $P = 0.0001$, compared to the baseline), and decreased in creatinine clearance. Rats receiving isolated IgG or serum without IgG from collapsing glomerulopathy developed mild proteinuria ($46.5 \pm 8.4$ mg/24 hours and $30.9 \pm 11$ mg/24 hours, respectively, at day 5 ($P = 0.0001$). Glomerular tuft retraction and podocyte damage were seen only in collapsing glomerulapthy rats. No abnormalities were found in rats injected with serum from FSGS or healthy subjects.

*Conclusion.* Circulating factors in the serum of collapsing glomerulopathy patients produce podocyte damage, whereas such factors are not present in noncollapsing FSGS. IgG eluates from collapsing glomerulopathy produce proteinuria when injected into the rat. Such factors remain in the circulation when serum of patients is adsorbed into protein A, raising the possibility that there are more than one circulating factor present in patients with collapsing glomerulopathy.

**Key words:** nephrotic syndrome, human circulating factors, collapsing glomerulopathy, focal segmental glomerulosclerosis.

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Collapsing glomerulopathy is an aggressive form of glomerular damage characterized by heavy proteinuria, renal insufficiency, and rapid progression to end-stage renal disease (ESRD) [1]. Collapsing glomerulopathy can be seen in association with human immunodeficiency virus (HIV) infection but it has been increasingly recognized in non-HIV patients as well [2]. In 1986, Weiss et al [1] reported six patients with rapidly progressive nephrotic syndrome and glomerular capillary collapse. Only one of the patients reported by Weiss subsequently developed aquired immunodeficiency syndrome (AIDS) and it was suggested that the rest of the patients could present a new clinicopathologic entity. Pathologic features were characterized by glomerular capillary collapse and visceral epithelial cell swelling along with tubular cystic dilatation and severe interstitial inflammatory changes [1, 2]. Epidemiologic findings show a strong predominance in the African American population [3]. Clinical findings include severe nephrotic syndrome (usually >10 g urinary protein in 24 hours), rapid progression to ESRD or death due to complications of nephrotic syndrome [2]. In order to make the diagnosis of primary collapsing glomerulopathy it is necessary to rule out the presence of HIV infection since collapsing glomerulopathy in HIV and non-HIV patients can be histopathologically indistinguishable [4]. Fifteen to fifty five percent of the patients with focal segmental glomerulosclerosis (FSGS) that undergo renal transplantation have recurrent disease [5, 6]. It has been suspected that circulating factors can play a role in the pathogenesis of visceral epithelial cell damage in steroid-resistant minimal change disease or recurrent FSGS [5]. Such factors are able to induce functional alterations in the renal tissue in vitro [6] and/or proteinuria in vivo when injected to the rat [5]. It is well known that patients with collapsing glomerulopathy present severe damage to the visceral epithelial cells that cause heavy proteinuria, usually >10 g in 24 hours [3]. Both recurrent and de novo collapsing glomerulopathy have been described in the renal allograft with

**Table 1.** Clinical characteristics of 10 patients with collapsing glomerulopathy

| Characteristic, patient number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Age *years* | 70 | 21 | 17 | 52 | 41 | 56 | 19 | 29 | 23 | 32 |
| Gender | F | F | F | F | M | M | M | M | M | M |
| Blood pressure *mm Hg* | 90/60 | 130/90 | 150/100 | 150/85 | 150/95 | 140/80 | 115/75 | 145/90 | 170/125 | 144/95 |
| Serum creatinine *mg/dL* | 1.6 | 7.3 | 2 | 1 | 0.8 | 2.9 | 0.9 | 1.53 | 1.7 | 1 |
| Blood urea nitrogen *mg/dL* | 15 | 25 | 24 | 22 | 9 | 29 | 14 | 32 | 32 | 18 |
| Creatinine clearance *mL/min*[a] | 29.6 | 5.8 | 25.5 | 47.6 | 96.3 | 26.4 | 105 | 54.2 | 37.1 | 82.6 |
| Urinary protein *g/day* | 15.00 | 10.00 | 14.00 | 12.00 | 12.70 | 10.00 | 11.00 | 10.00 | 12.30 | 14.00 |
| Cholesterol *mg/dL* | 269 | 134 | 418 | 341 | 362 | 246 | 213 | 363 | 239 | 226 |
| Triglycerides *mg/dL* | 103 | 162 | 737 | 181 | 452 | 327 | 120 | 243 | 107 | 132 |
| Serum albumin *g/day* | 1.9 | 0.9 | 1.4 | 2 | 2.1 | 4.6 | 3.4 | 4.3 | 1.6 | 3.3 |

[a]Creatinine clearance expressed in mL/min/1.73 $m^2$ as calculated by the Levey method (*Ann Intern Med* 130:461–470, 1999).

**Table 2.** Pathologic findings in 10 patients with collapsing glomerulopathy

| Patient number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Light microscopy | | | | | | | | | | |
| No. of glomeruli | 18 | 20 | 20 | 12 | 11 | 16 | 10 | 18 | 23 | 20 |
| Global collapse % | 10 | 0 | 10 | 25 | 10 | 25 | 0 | 10 | 20 | 0 |
| Segmental collapse % | 0 | 20 | 20 | 0 | 10 | 0 | 25 | 20 | 10 | 25 |
| Visceral epithelial cell hypertrophy damage % | 100 | 100 | 80 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Segmental sclerosis % | 0 | 10 | 0 | 0 | 0 | 0 | 10 | 0 | 0 | 0 |
| Global sclerosis % | 0 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 |
| Interstitial fibrosis[a] | 0 | I | I | 0 | I | I | I | II | I | II |
| Hyalinosis | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Tubular atrophy[a] | 0 | I | I | 0 | I | I | I | II | I | I |
| Tubular cysts[b] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | I | 0 | I |
| Immunofluorescence microscopy | Neg | 1 + IgM | Neg | Neg | Neg | Neg | Neg | Neg | 1 + IgM | Neg |
| Electron microscopy | ND | EE | FE | EE | EE | EE | ND | EE | FE | EE |

Abbreviations are: 1 + IgM, 1+/3+ mesangial deposition of IgM; ND, not done; EE, extensive visceral epithelial cell foot process effacement; FE, focal visceral epithelial cell foot process effacement.

[a]Scoring of interstitial fibrosis/atrophy 0, <10%; interstitial fibrosis/atrophy I, >10% <25% of cortical area; interstitial fibrosis/atrophy II, >25% <40% of cortical area; interstitial fibrosis/atrophy III, >40% of cortical area.

[b]Scoring of tubular cystic dilatation 0, negative; I, >10% <25% of cortical area.

histopathologic features similar to collapsing glomerulopathy in a native kidney [7]. Recurrence of collapsing glomerulopathy can occur hours after transplantation, raising the possibility that the plasma of collapsing glomerulopathy patients contains one or more factors capable of inducing proteinuria due to the damage of the visceral epithelial cell that results in the increase in glomerular permeability [7].

To explore the possibility that humoral factors are involved in the pathogenesis of the visceral epithelial cell damage in collapsing glomerulopathy patients, we injected serum and isolated IgG from patients with collapsing glomerulopathy to Sprague-Dawley rats in order to demonstrate that serum from patients with collapsing glomerulopathy is capable of producing proteinuria and ultrastructural changes in the visceral epithelial cells, similar to those abnormalities seen in patients with collapsing glomerulopathy, and compare it with serum from noncollapsing FSGS patients.

## METHODS

### Study subjects

We obtained serum from ten patients with a clinical and histopathologic diagnosis of collapsing glomerulopathy. All patients had nephrotic syndrome with proteinuria > 10 g/24 hours. Polymerase chain reaction (PCR) for parvovirus B-19 (PVB-19) was negative in all patients. Renal biopsy of patients with collapsing glomerulopathy presented characteristic glomerular capillary collapse, visceral epithelial cell swelling, and tubular damage. Direct immunofluorescence was negative for immune-complex deposition. Ultrastructural examination performed in all patients demonstrated diffuse foot processes obliteration. Three to four glomeruli were analyzed per case. Routinely, electron micrographs of five to six capillary loops were review at 10,000 magnifications after a low magnification of the whole glomerulus was performed. Detailed clinical data and pathologic findings are showed in Tables 1 and 2, respectively.

We also obtained serum from ten patients with nephrotic syndrome with idiopathic noncollapsing FSGS. All these patients fulfilled histopathologic criteria of FSGS not otherwise specified (NOS) according to the new pathologic classification of FSGS [8]. All biopsies presented discrete peripheral (nonperihilar or tip lesion) segmental consolidation of the glomerular tuft by increased extracellular matrix, with at least one glomerulus with segmental increase in matrix obliterating the capillary lumina. Cases belonging to the morphologic variants

**Table 3.** Clinical characteristics of 10 patients with focal segmental glomerulosclerosis (FSGS)

| Characteristic, patient number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Age *years* | 36 | 37 | 55 | 23 | 22 | 22 | 37 | 39 | 19 | 26 |
| Gender | M | M | M | F | F | F | F | M | M | F |
| Blood pressure *mm Hg* | 150/90 | 160/100 | 180/110 | 110/60 | 120/70 | 148/96 | 160/120 | 196/112 | 150/100 | 220/130 |
| Serum creatinine *mg/dL* | 2.2 | 1.5 | 1.2 | 2.5 | 0.7 | 2.5 | 2.5 | 1.5 | 3.0 | 0.9 |
| Blood urea nitrogen *mg/dL* | 27 | 22 | 22 | 32 | 18 | 19 | 27 | 22 | 29 | 16 |
| Creatinine clearance *mL/min*[a] | 37 | 52.4 | 59.3 | 25.5 | 71.4 | 26.9 | 23.5 | 56.7 | 29.6 | 81.1 |
| Urinary protein *g/day* | 5.7 | 8.3 | 3.4 | 3.0 | 6.0 | 3.5 | 3.1 | 3.0 | 5.4 | 3.1 |
| Cholesterol *mg/dL* | 202 | 273 | 260 | 270 | 645 | 269 | 287 | 272 | 283 | 297 |
| Triglycerides *mg/dL* | 195 | 202 | 184 | 260 | 685 | 305 | 282 | 252 | 208 | 154 |
| Serum albumin *g/dL* | 4.2 | 3.4 | 3.1 | 3.9 | 1.3 | 3.4 | 3.6 | 4.5 | 4 | 4.4 |
| Treatment | ACE | P, ACE | ACE | ACE | ACE | ACE | P, ACE | P, ACE | P, ACE | P, ACE |

Abbreviations are: P, pravastatine; ACE, angiotensin-converting enzyme inhibitor.
[a]Creatinine clearance expressed in mL/min/1.73 $m^2$ as calculated by the Levey method (*Ann Intern Med* 130:461–470, 1999).

**Table 4.** Pathologic findings in 10 patients with focal segmental glomerulosclerosis (FSGS)

| Patient number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Light microscopy | | | | | | | | | | |
| No. of glomeruli | 8 | 18 | 29 | 7 | 15 | 10 | 6 | 11 | 18 | 14 |
| Global collapse % | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Segmental collapse % | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Visceral epithelial cell prominence % | 25 | 100 | 30 | 30 | 100 | 100 | 100 | 20 | 100 | 30 |
| Segmental sclerosis % | 20 | 30 | 30 | 10 | 30 | 20 | 10 | 10 | 30 | 20 |
| Global sclerosis % | 10 | 0 | 10 | 0 | 0 | 10 | 0 | 0 | 10 | 0 |
| Interstitial fibrosis[a] | I | II | II | I | II | I | I | I | I | I |
| Hyalinosis % | 20 | 20 | 30 | 0 | 10 | 0 | 0 | 0 | 20 | 0 |
| Tubular atrophy[a] | I | II | II | 0 | I | I | I | I | I | I |
| Tubular cysts[b] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Immunofluorescence microscopy | Neg | 1 + IgM | Neg | Neg | Neg | Neg | Neg | Neg | 1 + IgM | Neg |
| Electron microscopy | ND | EE | FE | EE | EE | EE | ND | EE | FE | EE |

Abbreviations are: 1 + IgM, 1+/3+ mesangial deposition of IgM; ND, not done; EE, extensive visceral epithelial cell foot process effacement; FE, focal visceral epithelial cell foot process effacement.
[a]Scoring of interstitial fibrosis/atrophy 0, <10%; interstitial fibrosis/atrophy I, >10% <25% of cortical area; interstitial fibrosis/atrophy II, >25% <40% of cortical area; interstitial fibrosis/atrophy III, >40% of cortical area.
[b]Scoring of tubular cystic dilatation 0, negative; I, >10% <25% of cortical area.

with perihilar involvement, cellular variant, collapsing or tip lesion were excluded. Electron microscopy examination was performed in all cases. Three to four glomeruli were analyzed per case. Routinely, electron micrographs of five to six capillary loops were review at 10,000 magnification after a low magnification of the whole glomerulus was performed. Detailed clinical data and histopathologic findings are showed in Tables 3 and 4, respectively.

Serum from 12 healthy subjects from the Blood Bank, matched by age and gender, was used as a control. Serums of each group were pooled before fractionation.

### Fractionation of serum

Purified IgG from serum of healthy subjects, FSGS, and collapsing glomerulopathy patients was obtained by the alcoholic precipitation method [9]. Serum without IgG was obtained by fractionation with affinity-column with protein A agarose (Bio-Rad, Hercules, CA, USA) [5, 8, 9].

### Polyacrylamide gel electrophoresis (PAGE) of the proteins eluted from protein A

Proteins eluted from protein A agarose were resolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on SDS 5% to 8% gradient PAGE [5]. Proteins were electrophoretically transferred to nitrocellulose paper and immunoblotted against IgG to assure the molecular weight of the purified IgG obtained by both methods [10]. The protein bands and molecular weight standards were stained with Coomassie blue.

### Injection of serum and purified IgG into rats

Ninety-nine female Sprague-Dawley rats (250 to 280 g) (Charles Rivers, Madison, WI, USA) were randomly distributed and placed in metabolic cages (Nalgene) with water and food ad libitum. They were divided in 11 groups ($N = 9$): group 1, as control, received no injections; group 2 received saline solution (NaCl 0.9%); group 3 was given serum from healthy subjects, in its native form; group 4 received serum from collapsing glomerulopathy patients, in its native form; group 5 was given serum from non-collapsing FSGS patients, in its native form; group 6 received serum from healthy subjects without IgG; group 7 received serum from collapsing glomerulopathy patients without IgG; group 8 was given serum from FSGS without IgG; group 9 received purified IgG from healthy subjects; group 10 received purified IgG from collapsing

glomerulopathy patients; and group 11 was injected with purified IgG from FSGS patients.

Serum was administered once intravenously, daily, in the vein of the tail for 5 days in a concentration of 25 mg/mL in saline to a final volume of 1 mL, previous sedation with ether in a narcosis chamber (Daigger).

Urine was collected every 24 hours for 6 days: prior the first injection (baseline, day 0), and 24, 48, 72, 96, and 120 hours after the first injection (days 1 to 5, respectively). Proteinuria was measured using the method of Biurett described by Gornall, Bardawill, and David [11]. Urine of day 5 was also used for urinary creatinine determination by the method of Jaffé [12]. Rats were sacrificed 24 hours after the last injection. Blood samples were taken for creatinine determination in serum creatinine [12]. Left kidney was obtained fresh and kept frozen for immunohistochemical analysis. Right kidney was perfused-fixed in situ with a solution of 2% paraformaldehyde and 2.5% glutaraldehyde in cacodylate buffer (pH 7.4) for light and electron microscopy.

### Histopathologic analysis

Rats were sacrificed 24 hours after the last injection. Renal tissue was processed for light microscopy, direct immunofluorescence, and electron microscopy, according to standard techniques [13]. Sections for light microscopy were stained with hematoxylin and eosin, periodic acid-Schiff stain (PAS), Mallory trichrome stain and Jones' methenamine silver reaction. An average of 50 glomeruli per level of section in each sample was examined. All samples were evaluated for the presence of glomerular collapse, mesangial expansion, and/or prominence of the visceral epithelial cells. The presence of immune deposits was evaluated by direct immunofluorescence. Frozen renal tissue was stained with FICT-bound monoclonal antibodies against rat IgG (Caltag). Thirty to thirty five glomeruli were examined per case. Fluorescence intensity was evaluated in a semiquantitative form in a scale of 0 to 3+ (0, negative, 1+, weak, 2+, moderate, and 3+, strong). Electron microscopy examination was performed in all cases to look for the presence of tubuloreticular structures within the cytoplasm of endothelial cells of glomerular capillary loops and capillaries of the interstitium. Three to five glomeruli of each rat were examined. At the ultrastructural level we also analyzed wrinkling of the capillary loop basement membrane, visceral epithelial cell foot processes obliteration, and the presence of droplets and vacuoles within the podocytes. Renal biopsies were reviewed by an experienced renal pathologist (M.C.A.C.). Evaluation of slides was blinded to the observer.

### Statistical analysis

The results of the protocol of injections of serum and IgG from healthy subjects, not collapsing FSGS and collapsing glomerulopathy patients are expressed as the mean (±SD) of 24-hour proteinuria of groups of nine rats during the 5 days of injection period and the baseline (day 0). The statistical significance was calculated by the *t* test (Sigma Plot Stat, version 2.1). *P* value was set at $<0.05$, two-tailed.

## RESULTS

### Patients with collapsing glomerulopathy

Patients selected for the study fulfilled diagnostic criteria for collapsing glomerulopathy [1]. All patients agreed to participate in the study. Protocol was registered and approved by the Research and Ethics Review Board from the hospital. All patients were Mexican, serology for HIV was negative, and they denied contact with drugs. Patients had primary disease without family history of renal disease. Six collapsing glomerulopathy patients were males and four were females. Range of age varied from 17 to 70 years. At the time of diagnosis patients were hypertensive (blood pressure mean $144 \pm 28/95 \pm 27$ mm Hg). Serum creatinine was increased in six patients and varied from 0.8 to 7.3 mg/dL ($2.03 \pm 2$ mg/dL). All collapsing glomerulopathy patients presented massive proteinuria with urinary protein excretion > 10 g/24 hours. Foot processes effacement was confirmed by electron microscopy. It was diffusely present in all but one patient. This patient presented irregular foot process obliteration. Vacuoles and blebs within podocytes in all biopsies were also identified. Serum samples were taken from 1 week to 1 month after diagnosis was confirmed by a renal biopsy (Table 1).

### Patients with FSGS

Patients selected had nephrotic syndrome (Table 3) and a renal biopsy with diagnosis of noncollapsing FSGS (NOS), according to the new pathologic classification for the morphologic variants of FSGS [8]. All patients had primary disease without family history of renal disease. They were HIV negative and denied contact with drugs. Five patients were males and five were females. Range of age varied from 19 to 55 years. Eight out of 10 patients were hypertensive at the time of diagnosis. Serum creatinine varied from 0.7 to 2.5 mg/dL. All patients presented proteinuria in nephrotic ranges when serum samples were taken (between 1 and 4 weeks after the renal biopsy). Visceral epithelial foot process obliteration was present in six patients while two have irregular effacement. The presence of vacuoles and blebs within podocytes of all patients were also analyzed.

### Results of SDS-PAGE analysis of the protein eluted from protein A

SDS-PAGE of the protein eluted from protein A and of those obtained by alcoholic precipitation was used to

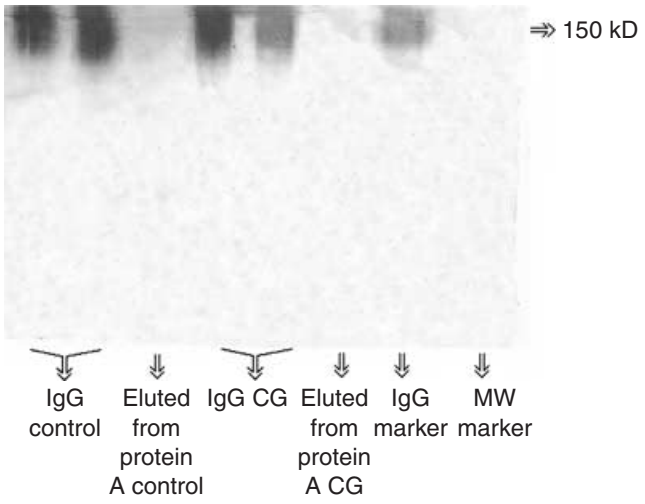


**Fig. 1. Results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the protein eluted from protein A.** The first two columns show the isolated immunoglobulins from healthy subjects (control) obtained by the alcoholic precipitation method. The third column presents the serum of healthy subjects without IgG, after protein A agarose fractionation. The fourth and fifth columns show isolated IgG from collapsing glomerulopathy (CG) patients obtained by the alcoholic precipitation method. The sixth column shows the serum of collapsing glomerulopathy patients without IgG, after protein A agarose fractionation. The seventh column shows a commercial IgG (150 dD) marker and the ninth column is a commercial molecular weight (MW) marker without IgG.

assess the molecular weight of the protein obtained by such methods. The molecular weight of the protein obtained by both methods, presumably IgG, corresponded with the commercially available IgG (150 kD) as seen in Figure 1.

### Effects of the injection of total serum and IgG from collapsing glomerulopathy patients, FSGS patients, and healthy subjects on the renal function and urinary protein excretion in rats

Results of the determination of urinary and serum creatinine of each group were used to calculate the creatinine clearance in mL/min, to evaluate the renal function. Results showed that rats receiving serum from collapsing glomerulopathy patients had a significant decrease in creatinine clearance compared with rats receiving serum from healthy subjects or saline ($P \leq 0.0001$). The group of FSGS fractionated by agarose A affinity (group 8) had a lesser, but significant, decrease in creatinine clearance compared to the healthy subject rats (group 6) ($P \leq 0.05$). The rest of groups receiving FSGS did not show abnormalities in renal function. Results of creatinine clearance values are shown in Table 5.

Urinary protein excretion in rats injected with serum or isolated IgG from collapsing glomerulopathy patients showed to be statistically significant ($P = 0.0001$ and $P \leq 0.00001$, respectively) compared with rats that received serum from FSGS or healthy subjects (Table 6) (Fig. 2), as follows. In group 1 (no injections), levels of urinary protein excretion remained in the baseline (similar to day 0), during the 5 days. In group 2 (injection of saline), levels of urinary protein excretion remained in the baseline during the 5 days of observation period. Rats in group 3 (serum of healthy subjects) failed to develop proteinuria during the 5 days of observation period. Rats in group 4 (serum collapsing glomerulopathy) presented proteinuria 24 hours after the first injection ($50.0 \pm 6.3$ mg/day) and the urinary protein excretion increased to $99.2 \pm 42$ mg/24 hours at day 5, $P = 0.0001$, compared to the baseline. In group 5 (serum FSGS), levels of urinary protein excretion remained in the baseline during the 5 days of observation period. Group 6 (serum of healthy subjects without IgG) rats did not develop proteinuria and urinary protein excretion remained in the baseline at day 5. In group 7 (serum collapsing glomerulopathy without IgG), rats developed mild proteinuria at days 4 and 5 ($30.9 \pm 11$) ($P = 0.0001$) compared to the baseline. In group 8 (serum FSGS without IgG), no proteinuria was found. Group 9 (isolated IgG from healthy subjects) rats failed to develop proteinuria and urinary protein excretion remained in the baseline. Group 10 (isolated IgG from collapsing glomerulopathy) rats developed mild proteinuria at day 3 with a light increase in the urinary protein excretion at day 5 ($46.5 \pm 8.4$) ($P = 0.0001$) compared to the baseline. Finally, group 11 (isolated IgG from FSGS) rats did not develop proteinuria and urinary protein excretion remained in the baseline at day 5.

### Histopathologic features

In group 1 (no injections), there were no abnormalities either at light microscopy or electron microscopy examination (Fig. 3B). In group 2 (injection of saline), there were no abnormalities either at light or ultrastructural examination (Fig. 3C). In group 3 (serum of healthy subjects), light and electron microscopy failed to show abnormalities in the glomeruli of rats (Fig. 3D). Group 4 (serum collapsing glomerulopathy) rats injected with serum of collapsing glomerulopathy patients showed retraction of the glomerular tuft, visceral epithelial cell swelling, and segmental collapse of the capillary loops (Fig. 3E). Glomeruli appeared bigger than control. Electron microscopy demonstrated extensive foot processes obliteration (Fig. 4A). Tubuloreticular structures were not identified. There was slight tubular injury characterized by changes in the cytoplasm that appeared eosinophilic, and contained some vacuoles, especially around some collapsed glomeruli. Nevertheless, brush border and nuclei were preserved. Group 5 (serum FSGS) rats were without abnormalities at light and electron microscopy (Fig. 3F). In group 6 (serum of healthy subjects without

**Table 5.** Results of urinary creatinine excretion, serum creatinine values, and creatinine clearance calculation

| Group | Body weight | Urinary creatinine *mg/dL* | Serum creatinine *mg/dL* | Creatinine clearance *mL/min* |
|---|---|---|---|---|
| 1 (control) | 270 ± 1 g | $1.4 \times 10^{-4} \pm 1.5 \times 10^{-5}$ | $1.0 \times 10^{-6} \pm 4.0 \times 10^{-7}$ | 1.29 ± 0.09 |
| 2 (saline) | 277 ± 2 g | $1.5 \times 10^{-4} \pm 3.6 \times 10^{-5}$ | $1.1 \times 10^{-6} \pm 2.6 \times 10^{-7}$ | 1.01 ± 0.33 |
| 3 (serum healthy subjects) | 272 ± 2 g | $1.5 \times 10^{-5} \pm 3.2 \times 10^{-5}$ | $1.3 \times 110^{-6} \pm 4.1 \times 10^{-7}$ | 0.77 ± 0.15 |
| 4 (serum collapsing glomerulopathy) | 275 ± 2 g | $9.5 \times 10^{-5} \pm 2.5 \times 10^{-5}$ | $2.2 \times 10^{-6} \pm 5.9 \times 10^{-7}$ | 0.32 ± 0.09[a] |
| 5 (serum focal segmental glomerulosclerosis) | 271 ± 1 g | $2.3 \times 10^{-4} \pm 1.0 \times 10^{-5}$ | $1.3 \times 10^{-6} \pm 5.5 \times 10^{-8}$ | 0.56 ± 0.07 |
| 6 (serum healthy subjects no IgG) | 270 ± 1 g | $1.9 \times 10^{-4} \pm 3.2 \times 10^{-5}$ | $9.3 \times 10^{-7} \pm 2.1 \times 10^{-7}$ | 1.55 ± 0.40 |
| 7 (serum collapsing glomerulopathy no IgG) | 273 ± 1 g | $1.1 \times 10^{-4} \pm 2.5 \times 10^{-5}$ | $1.5 \times 10^{-6} \pm 2.3 \times 10^{-7}$ | 0.68 ± 0.19[a] |
| 8 (serum focal segmental glomerulosclerosis no IgG) | 278 ± 3 g | $1.2 \times 10^{-4} \pm 2.8 \times 10^{-5}$ | $1.2 \times 10^{-6} \pm 0$ | 0.86 ± 0.20[b] |
| 9 (IgG healthy subjects) | 275 ± 2 g | $1.8 \times 10^{-4} \pm 3.0 \times 10^{-5}$ | $1.4 \times 10^{-6} \pm 1.1 \times 10^{-7}$ | 1.20 ± 0.68 |
| 10 (IgG collapsing glomerulopathy) | 272 ± 1 g | $1.1 \times 10^{-4} \pm 1.8 \times 10^{-5}$ | $2.2 \times 10^{-6} \pm 3.7 \times 10^{-7}$ | 0.43 ± 0.09[b] |
| 11 (IgG focal segmental glomerulosclerosis) | 275 ± 2 g | $1.1 \times 10^{-4} \pm 2.5 \times 10^{-5}$ | $1.1 \times 10^{-6} \pm 0$ | 0.81 ± 0.21 |

[a] $P \leq 0.02$ and [b] $P \leq 0.05$ of collapsing glomerulopathy serum and focal segmental glomerulosclerosis serum versus healthy subjects. Results expressed in mean ± SD.

**Table 6.** Urinary protein daily excretion in the 11 groups of Sprague-Dawley rats[a]

| Group | Baseline *mg/day* | 24 hours *mg/day* | 48 hours *mg/day* | 72 hours *mg/day* | 96 hours *mg/day* | 120 hours *mg/day* |
|---|---|---|---|---|---|---|
| 1 (control) | 6.0 ± 4.8 | 6.7 ± 4.7 | 3.9 ± 4.7 | 6.2 ± 3.3 | 5.0 ± 4.0 | 2.6 ± 3.4 |
| 2 (saline) | 3.9 ± 3.5 | 5.6 ± 3.3 | 5.1 ± 3.03 | 5.4 ± 2.4 | 5.1 ± 3.2 | 5.0 ± 3.4 |
| 3 (S.HS) | 5.3 ± 3.4 | 4.6 ± 2.9 | 5.8 ± 3.1 | 7.3 ± 3.9 | 9.1 ± 2.7 | 4.1 ± 3.1 |
| 4 (S CG) | 13.5 ± 6.4 | 50.0 ± 6.3 | 57.0 ± 15 | 62.3 ± 16 | 90.5 ± 19 | 99.2 ± 42 |
| 5 (S FSGS) | 0 | 0 | 4.6 ± 2.9 | 6.4 ± 0 | 16.9 ± 2 | 13.08 ± 0 |
| 6 (S HS no IgG) | 0 | 0 | 0 | 3.9 ± 5.2 | 6.2 ± 6.1 | 7.2 ± 6.1 |
| 7 (S CG no IgG) | 8.8 ± 7.3 | 16.8 ± 12 | 17.1 ± 11 | 25.4 ± 7.4 | 30.5 ± 4.3 | 30.9 ± 11 |
| 8 (S FSGS no IgG) | 0 | 0 | 0 | 2.9 ± 3.3 | 11.5 ± 13 | 11.5 ± 11 |
| 9 (IgG CG) | 0 | 11.3 ± 3.9 | 26.0 ± 3.4 | 38.7 ± 5.8 | 42.7 ± 9.2 | 46.5 ± 8.4 |
| 10(IgG HS) | 4.6 ± 4.9 | 4.5 ± 4.3 | 5.8 ± 5.5 | 5.4 ± 5.4 | 0 | 0 |
| 11(IgG FSGS) | 0 | 0 | 0 | 0 | 0 | 8.0 ± 9.3 |

Abbreviations are: S.HS, serum healthy subjects; S GC, serum collapsing glomerulopathy; S.HS no IgG, serum of healthy subjects without IgG; S. CG no IgG, serum of collapsing glomerulopathy without IgG, IgG HS, isolated IgG of healthy subjects; IgG CG, isolated IgG of collapsing glomerulopathy; IgG FSGS, isolated IgG of focal segmental glomerulosclerosis.

[a] Mean ± SD.

IgG), there were no abnormalities at light or electron microscopy (Fig. 3G). Group 7 (serum collapsing glomerulopathy without IgG) rats showed prominence of visceral epithelial cells and segmental capillary retraction. No tubular damage was found. Ultrastructural examination demonstrated foot processes obliteration (Fig. 3H). In group 8 (serum FSGS without IgG), there were no glomeruli abnormalities at light or electron microscopy (Fig. 3I). Group 9 (isolated IgG of healthy subjects) rats showed no abnormalities at light or electron microscopy (Fig. 3J). Group 10 (isolated IgG collapsing glomerulopathy) rats injected with isolated IgG from patients with collapsing glomerulopathy showed retraction of the glomerular tuft with prominence of the visceral epithelial cells (Fig. 3K). Tubular damage around retracted glomeruli was not identified. Ultrastructural examination demonstrated irregular foot process obliteration. Tubuloreticular structures were not found. Finally, in group 11 (isolated IgG from FSGS), there were no abnormalities at light or electron microscopy (Fig. 3L). Visceral epithelial cell foot process obliteration of groups injected with collapsing glomerulopathy serum is showed in Figure 4 (Table 7).

Direct immunofluorescence against rat IgG was negative for the presence of immune-complex in all groups. Linear IgG against glomerular basemen membrane was seen in groups receiving collapsing glomerulopathy serum (groups 4, 7, and 10) (data not shown).

## DISCUSSION

In the present study we injected serum from patients with clinical and pathologic diagnosis of collapsing glomerulopathy to Sprague-Dawley rats in order to study the effect of the injection on the production of proteinuria and histopathologic damage, in vivo, compared with the injection of serum from FSGS patients and healthy subjects. Serum was administered in one of three forms: (*1*) in its native form (nonadsorbed), (*2*) only the isolated IgG obtained by alcoholic precipitation method, and (*3*) serum without IgG (extracted by chromatography with protein A agarose). Results showed clearly that the injection of serum of collapsing glomerulopathy patients to Sprague-Dawley rats in any of these three forms induces proteinuria and visceral epithelial cell damage.

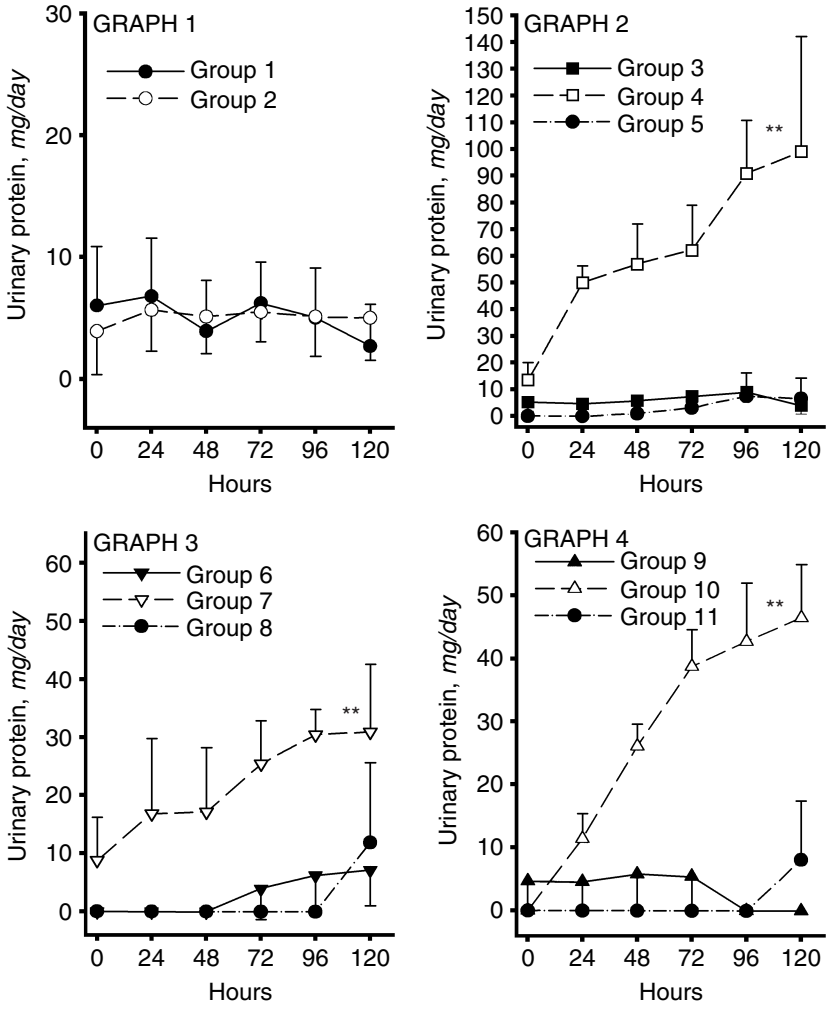


**Fig. 2. Urinary protein excretion in rats.** Graph 1 shows urinary protein excretion of rats of groups 1 (no injections) and 2 (injections of saline), remained in the baseline (<10 mg/day) during the 5 days of observation period. Graph 2 compares the urinary protein excretion between groups 3 and 4 and group 5. While urinary protein excretion of rats receiving serum of healthy subjects and focal segmal glomerulosclerosis (FSGS) in its native form (groups 3 and 5) remains in the baseline, rats receiving serum of collapsing glomerulopathy patients in its native form (group 4) present proteinuria 24 hours after the first injection (50.0 ± 6.3 mg/day) and finally to 99.2 ± 42 mg/24 hours at day 5 (**$P = 0.0001$ compared to its baseline). Graph 3 shows rats of groups 6 (serum of healthy subjects without IgG) and 8 (serum from FSGS without IgG) did not develop proteinuria and urinary protein excretion remained in the baseline at day 5. Rats of group 7, (serum collapsing glomerulopathy without IgG) developed proteinuria 24 hours after the first injection and mild proteinuria was reached at days 4 and 5 (30.9 ± 11) (**$P = 0.0001$ compared to its baseline). Graph 4 shows rats of groups 9 (isolated IgG of healthy subject) and 11 (isolated IgG of FSGS) failed to develop proteinuria at day 5 compared to rats of group 10, (isolated IgG of collapsing glomerulopathy) that developed mild proteinuria at day 3 with a light increase in the urinary protein excretion at day 5 (46.5 ± 8.4) (**$P = 0.0001$ compared to its baseline).

It has been proposed that patients with damage to the visceral epithelial cell with a pattern of minimal change disease and FSGS with recurrence of nephrotic syndrome in the allograft could present a circulating factor responsible for the production of proteinuria [5, 6, 14, 15]. An elegant in vitro study has demonstrated that some circulating factors present in patients with FSGS can produce changes in glomerular permeability when isolated glomeruli are incubated with the serum of the patients [14]. Another study has demonstrated that a factor present in the plasma of FSGS patients increases glomerular permeability in vitro and causes transient proteinuria in vivo [15, 16]. However, up to now, there are no in vivo studies clearly demonstrating the evidence of histopathologic damage to the visceral epithelial cell secondary to the presence of circulating factors exclusively in collapsing glomerulopathy. To support the evidence of a circulating factor present in patients with collapsing glomerulopathy it has been pointed out that collapsing glomerulopathy can recur rapidly after transplantation [15]. Reported time for recurrence in the allograft varies between 4 and 26 weeks [15, 16], based on the presence of proteinuria, although this fact depends also on the time performance of the biopsy since some reports have found recurrence of the disease as far as 144 months after transplantation, when a renal allograft biopsy has been performed [7]. The hypothesis of circulating factors has also been supported by the observation that plasmapheresis and plasma protein adsorption onto a protein A column often decrease urinary protein excretion in patients with recurrent idiopathic nephrotic syndrome [16, 17]. The effect on the urinary protein excretion is only transient and after some weeks patients may have recurrence of the symptoms [5, 16]. Therefore, the possibility that patients with recurrent FSGS have circulating factors that are capable to alter glomerular permeability to macromolecules has been suggested repeatedly by different authors [2, 5, 6, 14, 16].

Studies in the utility of plasmapheresis in the treatment of recurrent FSGS have shown that this method reduces proteinuria and glomerular injury [17]. In the former study, plasmapheresis reduced proteinuria from a mean of 12 ± 7.46 g/24 hours to 5.1 ± 7.43 g/24 hours [17]. The demonstration of circulating factors in the pathogenesis

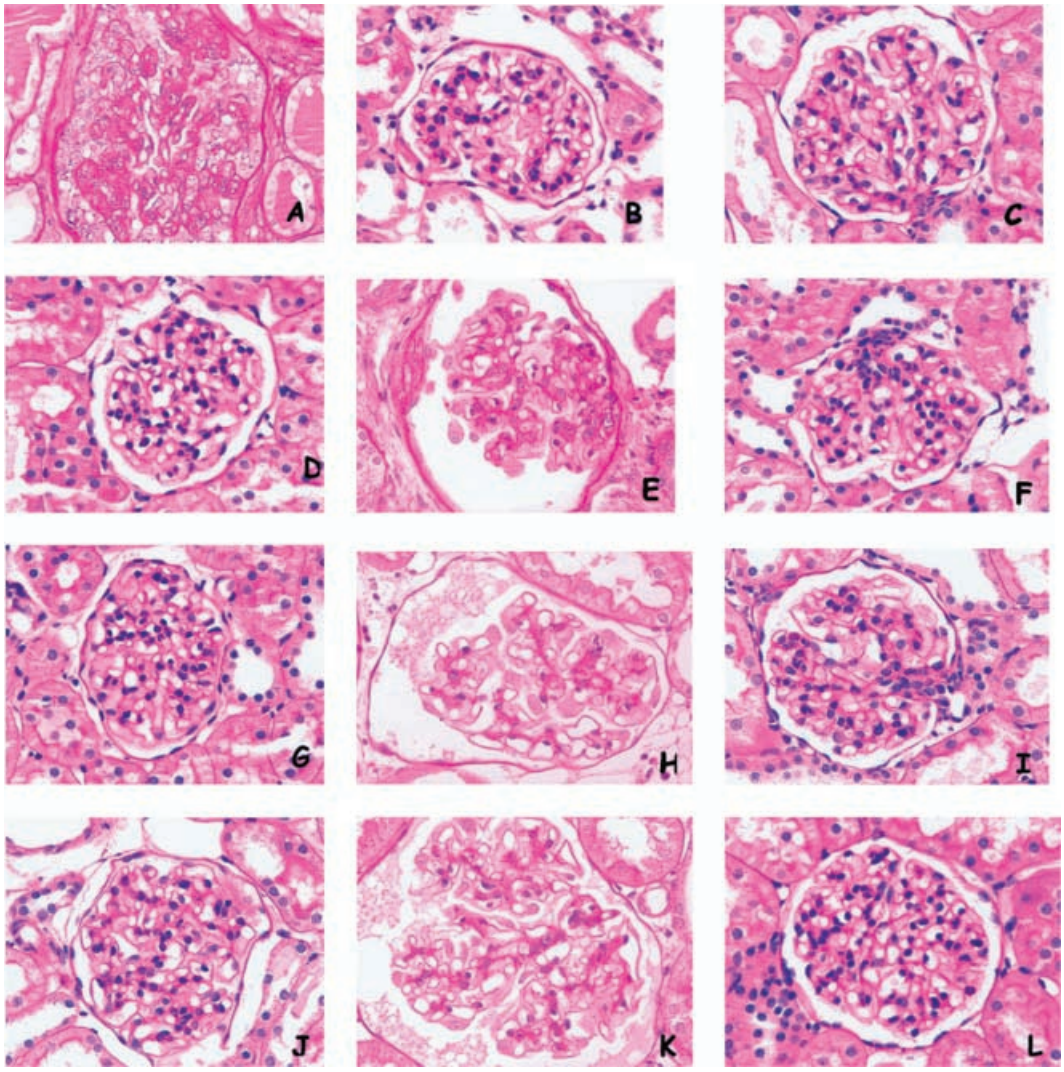


**Fig. 3. Histopathologic features.** (*A*) Light microscopy of a patient with diagnosis of collapsing glomerulopathy. Retraction and collapse of the glomerular tuft is seen. Visceral epithelial cells appear hypertrophied in the urinary space (periodic acid-Schiff stain 400×). (*B*) Group 1 (no injections). No abnormalities are seen at light microscopy. Capillary loops are open (periodic acid-Schiff stain 600×). (*C*) Group 2 (injection of saline). No abnormalities are seen at light microscopy. Capillary loops are open (periodic acid-Schiff stain, 600×). (*D*) Group 3 (serum of healthy subjects). Light microscopy failed to show abnormalities in the glomeruli of rats belonging this group. Capillary loops are open and capillary loops basement membranes are fine and delicate. Visceral epithelial cells are not appreciated (periodic acid-Schiff stain, 600×). (*E*) Group 4 (serum collapsing glomerulopathy patients). Glomerulus is large. Global retraction of the glomerular tuft, visceral epithelial cell swelling and collapse of the capillary loop are appreciated (periodic acid-Schiff stain, 600×). (*F*) Group 5 [serum focal segmental glomerulosclerosis (FSGS) patients]. Glomerulus appears normocelular, capillary loops are open. Visceral epithelial cells are not appreciated (periodic acid-Schiff stain, 600×). (*G*) Group 6 (serum of healthy subjects without IgG). No abnormalities at light microscopy are present in this group (periodic acid-Schiff stain, 600×). (*H*) Group 7 (serum collapsing glomerulopathy without IgG). Glomerulus is large. Prominence of visceral epithelial cells and segmental capillary retraction are present in this rats. Although brush border is preserved, some vacuoles are seen within tubular cells (periodic acid-Schiff stain, 600×). (*I*) Group 8 (serum FSGS without IgG). Capillary loops are open, no abnormalities are seen by light microscopy (periodic acid-Schiff stain, 600×). (*J*) IgG of healthy subjects. No abnormalities at light microscopy were found in this group. Capillary loops are open (periodic acid-Schiff stain, 600×). (*K*) Isolated IgG collapsing glomerulopathy. Glomerulus is large. Partial retraction of the glomerular tuft with prominence of the visceral epithelial cells (periodic acid-Schiff stain, 600×). (*L*) Isolated IgG FSGS. Capillary loops are open (periodic acid-Schiff stain, 600×).

of the glomerular damage in collapsing glomerulopathy would be very useful, since treatments like plasmapheresis could help not only to reduce proteinuria but, if such treatment is initiated early after diagnosis, patients could also have lasting remissions.

Although there has been no evidence of the involvement of immunoglobulins in the pathogenesis of collapsing glomerulopathy, some studies have proposed that IgG could have a role in the production of proteinuria in recurrent nephrotic syndrome in FSGS [5]. These studies have demonstrated a decrease in urinary protein excretion in patients with recurrent nephrotic syndrome after protein adsorption with protein A. It is well known that protein A binds the constant domains of some immunoglobulins [5].

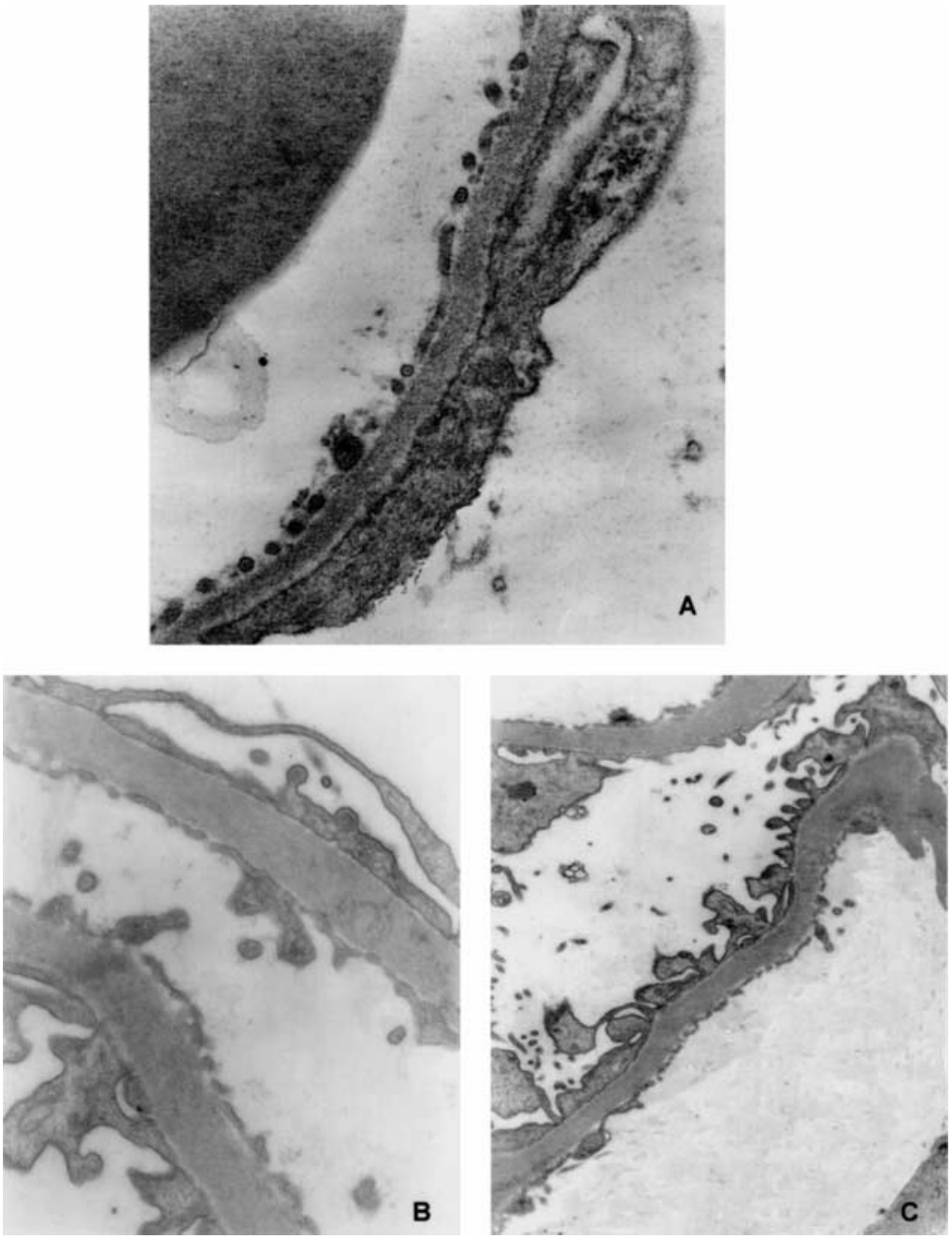


**Fig. 4. Electron micrographs.** (*A*) Ultrastructural examination of rat of group 4 (serum collapsing glomerulopathy) showing extensive foot process obliteration (20,000×). (*B*) Capillary loop of rat of group 7 showing irregular visceral epithelial cell foot process effacement (20,000×). (*C*) Capillary loop of rat of group 10 showing irregular foot process effacement (6300×).

In the present study, rats injected with purified IgG from patients with collapsing glomerulopathy (group 10) developed mild proteinuria (46.5 ± 8.4 mg/day), compared with those rats injected with serum from patients with collapsing glomerulopathy in its native form (group 4) that presented heavy proteinuria at day 5 (99.2 ± 42 mg/day). What is more, rats injected with serum from patients with collapsing glomerulopathy without IgG (group 7), also presented mild proteinuria (30.9 ± 11 mg/day). This observation may suggest that the factors responsible for the production of proteinuria are not only bound to the IgG, and that those other factors present in the rest of the serum may also play an important role in the production of proteinuria. It can also stress the fact that there is more than one factor responsible for the alteration of the glomerular permeability, and that the administration of all factors together in the serum in its native form, as seen in rats of group 4, produces more proteinuria than when

**Table 7.** Histopathologic findings in 11 groups

| Group | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Light microscopy | | | | | | | | | | | |
| No. of glomeruli | 120 | 137 | 143 | 144 | 130 | 123 | 140 | 144 | 150 | 138 | 130 |
| Global retraction % | 0 | 0 | 0 | 25 | 0 | 0 | 20 | 0 | 0 | 20 | 0 |
| Segmental collapse % | 0 | 0 | 0 | 20 | 0 | 0 | 20 | 0 | 0 | 20 | 0 |
| Visceral epithelial cell prominence % | 0 | 0 | 0 | 50 | 0 | 0 | 30 | 0 | 0 | 25 | 0 |
| Segmental sclerosis % | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Global sclerosis % | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Interstitial fibrosis[a] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Tubular necrosis | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Immunofluorescence | Neg | Neg | Neg | Neg, linear | Neg | Neg | Neg, linear | Neg | Neg | Neg, linear | Neg |
| Electron microscopy | N | N | N | EE | N | N | FE | 0 | 0 | FE | N |

Abbreviations are: N, normal; EE, extensive visceral epithelial cell foot process effacement; FE, focal and irregular visceral epithelial cell foot process effacement. No. of glomeruli represents the average of glomeruli per section. Glomerular damage was present only in rats receiving serum from CG patients.

those factors are administered individually (groups 7 and 11). It has been found that protein A eluates contain not only immunoglobulin but also other proteins [5]. Proteins eluted from protein A agarose were analyzed by SDS-PAGE [5] on 5% to 10% polyacrylamide gradient gels under reducing and nonreducing conditions to assure the molecular weight of the purified IgG. Immunoblotting of these gels showed a band of protein identical to the band of commercial IgG, used as a control, and we did not find evidence of other bands of proteins. It is important to stress out the fact that our groups injected with serum from patients with noncollapsing FSGS (groups 5, 8, and 11) did not develop proteinuria or visceral epithelial cell damage by light or electron microscopy. Studies demonstrating the presence of circulating factors in patients with nephrotic syndrome or recurrent nephrotic syndrome in the allograft were performed in patients diagnosed as steroid resistant FSGF or recurrent FSGS [5]. Several of those studies show minimal or no depiction of the cases from which serum was derived, neither the morphologic variant of FSGS, especially if collapsed glomeruli were present in the biopsy [5, 16–18]. In the present study we utilized serum only from prospectively selected patients with noncollapsing FSGS classified as FSGS (NOS) according to the recently proposed classification for the morphologic variants of FSGS [8]. All patients had nephrotic range proteinuria by the time serum samples were taken. Many other studies showing permeability factors in FSGS have been performed in vitro and not as in vivo assay [16]. Therefore, another explanation could be that noncollapsing FSGS patients have different factors that collapsing glomerulopathy patients and they could be weakened by degradation when administrated to the rat by intravenous injection in the vein of the tail, resulting in an absence of response. Another important possibility to point out is that data are real and there are only certain morphologic variants of FSGS with circulating factors that alter the glomerular filtration.

The histopathologic features of the different groups of rats showed clearly the presence of visceral epithelial cell damage in all rats receiving serum from patients with collapsing glomerulopathy compared with those receiving serum from FSGS or healthy subjects. There was no difference in the type of damage between all groups that received serum from collapsing glomerulopathy patient (groups 4, 7, and 11), but in group 4 (serum of collapsing glomerulopathy patients in its native form) there was extensive foot processes obliteration by electron microscopy; this correlates with major proteinuria in this group. It is interesting that glomeruli of collapsing glomerulopathy rats in all groups appear diffusely bigger than controls. Although there was no striking damage to the tubules, eosinophilia in proximal tubules, especially those surrounding some collapsed glomeruli, was identified. This change could be the result of tubular damage due to proteinuria since droplets of albumin can be observed in these areas. Light microscopy of all rats injected with collapsing glomerulopathy sera had some retraction of the glomerular tuft but we did not find marked glomerular collapse and droplets within the visceral epithelial cells. The presence of wrinkling of the glomerular basement membrane or tubuloreticular structures in the endothelium of the capillary loops were not found by electron microscopy examination in any of the cases. Therefore, differing from noncollapsing FSGS rats, animals injected with serum from patients with collapsing glomerulopathy developed a characteristic damage of the visceral epithelial cells and proteinuria, as seen in collapsing glomerulopathy patients. One important point is that in all previous reported studies there is a lack of relation between the specific morphologic type of visceral epithelial cell disease and the plasma factors implicated, since the morphologic and clinical characteristics of the patients are not provided and they are all included under the term recurrent FSGF or recurrent nephrotic syndrome [14, 19–21]. Our study was performed with sera of patients with clinical and pathologic diagnosis of primary collapsing glomerulopathy, exclusively, versus sera from patients with noncollapsing FSGS.

The precise characterization of the biochemical properties and nature of the circulating factors implicated in the development of glomerular damage and proteinuria in

collapsing glomerulopathy will need further studies. An inconsistent proteinuria following the injection of FSGS sera into experimental animals has been attributed to factors present in normal serum that block the permeability effect of active FSGS sera [20]. Although different studies on the characterization of plasma factors has been performed [13, 19–21], at the present time, the precise nature of the active factors remains speculative [22]. As a consequence, the relationship between the permeability factor and glomerular capillary collapse and scarring has not been clarified. We have enough evidence to support the fact of the presence of circulating factors that produce proteinuria and visceral epithelial damage in collapsing glomerulopathy patients. Differing from collapsing glomerulopathy, serum from noncollapsing FSGS did not produce visceral epithelial cell damage when injected to the rat raising the possibility that permeability substances in plasma of FSGS patients are present only in certain variants of this striking disease.

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