Apolipoproteins Prevent Glomerular Albumin Permeability Induced *In Vitro* by Serum from Patients with Focal Segmental Glomerulosclerosis

GIOVANNI CANDIANO,* LUCA MUSANTE,* MICHELE CARRARO,† LUIGI FACCINI,† LUCIANO CAMPANACCI,† CRISTINA ZENNARO,† MARY ARTERO,† FABRIZIO GINEVRI,* FRANCESCO PERFUMO,* ROSANNA GUSMANO,* and GIAN MARCO GHIGGERI*

**Unit and Laboratory of Nephrology, Istituto G. Gaslini, Genoa, and †Istituto di Medicina Clinica, University of Trieste, Trieste, Italy.*

Abstract. Glomerular albumin permeability alterations can be induced *in vitro* by serum from patients with end-stage renal disease caused by primary focal segmental glomerulosclerosis (FSGS). It was hypothesized that inhibitory substances may be present in normal serum, which may prevent the permeability alterations in isolated glomeruli, and the present study sought to isolate and characterize these factors. Albumin permeability was determined from the change in glomerular volume induced by applying oncotic gradients across the basement membrane of healthy isolated rat glomeruli preincubated with FSGS serum and normal serum fractionated using standard techniques. Fractions of normal serum with inhibitory activity obtained by a multistep chromatographic procedure underwent two-dimensional electrophoresis and staining. Approximately 50 protein spots were recovered, renatured, and tested for antipermeability activity. Five of these proteins demonstrated consistent inhibitory activity, and desorption ionization and mass spectrometry proved them to be components of high-density lipoprotein: apolipoproteins (apo) E_2 and E_4, high-molecular-weight J and L, and a 28-kD fragment of A-IV. Polyclonal antibodies to apo E or apo J added to the whole normal serum restored the permeability activity of the FSGS serum in the bioassay. Commercially available apo E and apo J also demonstrated antipermeability activity when added to FSGS serum. Cyanogen bromide digestion of apo A-IV produced fragments that inhibited the permeability activity of the FSGS serum, whereas the intact protein did not. Thus, components of high-density lipoprotein are capable of preventing glomerular albumin permeability induced by serum from patients with FSGS in an *in vitro* system. The specificity and mechanism of the inhibition remain to be determined; the alteration of normal inhibitory activity *in vivo* may be a component in the pathophysiology of FSGS.

The appearance of nephrotic range proteinuria after renal transplantation in approximately 30% of patients previously diagnosed with primary focal segmental glomerulosclerosis (FSGS) (1) remains unexplained. Several investigators hypothesized that a circulating humoral factor is involved in the pathogenesis, based on the efficacy of *ex vivo* techniques including plasmapheresis and immunoabsorption in reducing proteinuria (2–4) and on indirect laboratory evidence provided by *in vitro* bioassays that detect albumin permeability (P_{alb}) changes induced by patient serum in isolated normal glomeruli (5,6). For example, Dantal and colleagues (4) found that protein excretion associated with recurrent nephrotic syndrome could be reduced by treating the patients with cycles of protein adsorption on protein A columns. Concentrated eluates from the protein A columns of some patients provoked proteinuria when injected intravenously into laboratory rats. The same group of investigators further characterized the putative permeability factor using anti-Ig immunoaffinity columns (7). Their results suggest that the permeability factor may be an Ig or a substance bound to an Ig. Sharma *et al.* (8) recently characterized the biochemical properties of a permeability factor, apparently a 30- to 50-kD protein, found in the plasma of 15 FSGS patients using the isolated glomeruli technique. Also, these investigators successfully induced proteinuria in rats after a latency period of 6 to 24 h, injecting intravenously the enriched plasma supernatants.

Thus, the putative humoral mediator thought to be involved in the pathogenesis of idiopathic FSGS, although it has not yet been identified, may be categorized with other serum factors that have the potential under pathologic conditions to damage, directly or indirectly, the glomerular permeability barrier, such as the terminal attack complex of complement, superoxide radicals, and serine proteases. However, serum also contains substances—superoxide dismutase, protease inhibitors, etc.—

G.C. and L.M. contributed equally to the work.

Received April 14, 2000. Accepted June 18, 2000.

Correspondence to Dr. Gian Marco Ghiggeri, Unit and Laboratory of Nephrology, Istituto G. Gaslini, 16148 Genova, Italy. Phone: 0039 010 380742; Fax: 0039 010 395214; E-mail: labnefro@ospedale-gaslini.ge.it

1046-6673/1201-0143

Journal of the American Society of Nephrology

that normally limit or block the activity of agents that can provoke glomerular injury.

Savin *et al.* (9) observed that the addition of normal sera from a variety of species to FSGS sera effectively inhibited permeability alterations in isolated glomeruli. Thus, normal serum may contain particular factors that limit the injury induced by FSGS serum, at least in isolated glomeruli *in vitro*. Our objective in the present study was to isolate and characterize these factors as a novel approach to understanding the pathogenesis of the idiopathic nephrotic syndrome.

Materials and Methods

Sera

Sera from three children (two males, age range 5 to 10 yr) who had received a diagnosis of FSGS that recurred after a first renal transplantation and who were found to be highly positive for the putative P_{alb} factor (>0.9, see below) were used separately as a positive control. The diagnosis of FSGS was based on clinical and pathologic criteria, including nephrotic range proteinuria, unresponsiveness to steroids, and glomerular focal segmental sclerosis on transplant biopsy. Proteinuria recurred within 2 mo of transplantation in all three children. Pooled normal serum from 100 healthy blood donors (approximately 1 L) was used for the isolation of antipermeability factors.

Isolation of Glomeruli and Calculation of P_{alb}

The glomeruli were isolated from healthy male Sprague-Dawley rats that weighed 200 to 300 g abd that had been maintained in the laboratory animal facility of the University of Trieste, according to the humane treatment guidelines established by the university. The animals were anesthetized with ether and decapitated, and the kidneys were excised. The glomeruli were extracted from the minced renal cortex by standard sieving techniques in medium containing 115 mmol/L sodium chloride, 5 mmol/L potassium chloride, 10 mmol/L sodium acetate, 1.2 mmol/L dibasic sodium phosphate, 25 mmol/L sodium bicarbonate, 1.2 mmol/L magnesium sulfate, 1.0 mmol/L calcium chloride, and 5.5 mmol/L glucose. The pH had been titrated to 7.4. The medium also contained 5 g/dl bovine serum albumin as an oncotic agent. During the sieving process, the glomeruli had been stripped from Bowman's capsule and their tubules and associated blood vessels. The isolated glomeruli were then washed in 1 ml of fresh medium, and an aliquot of 0.1 ml was incubated at 37°C for 10 min in 0.9 ml of medium that included either 2 to 4% vol/vol FSGS serum and the normal serum fraction, as described below, or pooled normal human serum, which served as the control. The glomeruli were then plated onto a glass coverslip, coated with poly-L-lysine as an adherent, and covered with fresh medium. The samples were masked to eliminate operator bias.

The rationale and methodology for the determination of P_{alb} has been described in detail in the literature (5,10). In brief, each of 10 to 16 glomeruli per test serum were videotaped through an inverted microscope before and after a medium exchange to one containing 1 g/dl bovine serum albumin. The medium exchange created an oncotic gradient across the basement membrane, resulting in a glomerular volume change ($\Delta V = [V_{final} - V_{initial}]/V_{initial}$) that was measured off-line by a video-based image analysis program (MCID, Imaging Research Inc., St. Catharines, Ontario, Canada). The magnitude of ΔV was related to the albumin reflection coefficient, σ_{alb}, by the following equation:

$$(\sigma_{alb})_{experimental} = (\Delta V)_{experimental}/(\Delta V)_{control}$$

The σ_{alb} of the control glomeruli was assumed to be equal to 1. P_{alb} is defined as $(1 - \sigma_{alb})$ and describes the movement of albumin subsequent to water flux. When σ_{alb} is 0, albumin moves across the membrane with the same velocity as water, and P_{alb} is 1.0. Conversely, when σ_{alb} is 1.0, albumin cannot cross the membrane with water, and P_{alb} is 0.

Effects of FSGS Serum and FSGS Serum and Whole Normal Serum on Glomerular P_{alb}

To confirm elevated P_{alb} values from the FSGS serum samples, the average P_{alb} was determined during three separate analyses from each of the three patients. On the basis of data from the literature (5), P_{alb} values > 0.5 in patients with recurrent FSGS were considered significantly elevated compared with control populations. To assess the effect of normal serum on the ability of FSGS serum to alter glomerular P_{alb}, normal serum (20 μl) was added to the FSGS serum from each of the three patients in a 1:1 ratio before incubation with the rat glomeruli. Data were expressed as mean ± SEM.

Isolation Procedure

In preliminary studies, the pooled normal serum was passed through a number of different resin chromatography columns, including ionic exchange, hydroxyapatite, gel filtration, and pseudo-affinity, before the hydrophobic nature of the antipermeability activity was recognized. Thereafter, the pooled normal serum was precipitated with ammonium sulfate (40 to 60%), and after centrifugation the pellet was applied in aliquots to a 30 × 2.5-cm column of phenyl-sepharose (Pharmacia Amersham, Little Chalfont, UK) equilibrated with two column volumes of 1.5 M ammonium sulfate in 25 mM phosphate buffer (pH 7.2) to enrich the yield. After a rapid gradient in which the concentration of ammonium sulfate decreased from 1.5 to 0 M, proteins with inhibitory activity in the bioassay were eluted with ethanol 20% and then mixed with n-buthanol (1:5 vol/vol), which produced a three-layer phase: a bottom aqueous layer, an intermediate colloidal layer, and an upper layer containing the buthanol. The middle layer, which contained the maximum inhibitory activity, was extracted in acetonitrile 80%/trifluoracetic acid 0.1% (11), and the pellet was then extracted again in guanidine HCl 6 M overnight at 4°C. The resulting extract was then re-equilibrated in 8 M urea and dialysed before two-dimensional electrophoresis.

Two-Dimensional Electrophoresis and Protein Staining

The pellet was further fractionated by two-dimensional electrophoresis, which consisted of immobilized pH gradients between 3 and 10 in the first dimension and sodium dodecyl sulfate (SDS) polyacrylamide in the second dimension, following the original technique described by Bjellqvist *et al.* (12). Methods for sample preparation, rehydration of immobilized pH gradients, and polyacrylamide electrophoresis have been described in detail (13). Proteins were identified by methyl trichloroacetate-negative staining (14). Gels for evaluating permeability activity and for peptide fragmentation were run in parallel, and spots from the two-dimensional gel were cut after negative staining. Copper and Coomassie R20 stainings were performed as described previously (13–15). For Western blot, proteins were transblotted to Hybond nitrocellulose membranes (Amersham Pharmacia Biotech) with a Novablot semidry system using a continuous buffer system with 38 mM Tris, 39 mM glycine, 0.035% SDS, and 20% methanol. The transfer was achieved at 1.55 mA/cm^2 for 3.5 h. For immunostaining, we used the following antibodies: (*1*) mouse anti-human apolipoprotein A-IV (apo A-IV) monoclonal antibodies (clone 6C2A7, Boehringer Mannheim, Mannheim, Germany), (*2*) polyclonal

goat anti-human apo J (Chemicon, Temecula, CA), and (*3*) polyclonal rabbit anti-human apo E (Dako, Copenhagen, Denmark). Donkey anti-goat, goat anti-mouse, and goat anti-rabbit antibodies conjugated with alkaline phosphatase (Biorad, Hercules, CA) were used as second antibodies.

Recovery and Renaturation of Proteins

Proteins for the bioassay were recovered from polyacrylamide gel spots by gentle pestling. After an initial equilibration with 250 mM Tris-0.5% ethylenediaminetetraacetate, proteins were incubated overnight with 0.1% SDS-2 mM ethylenediaminetetraacetate, and after centrifugation the supernatant was dialyzed against guanidine HCl 4 M, according to the technique described by Hager *et al.* (16). Dialysis was then continued against several changes of water for 24 h.

Matrix-Assisted Laser Desorption Ionization and Mass Spectrometry

The methods of in-gel digestion and matrix-assisted laser desorption ionization and mass spectrometry (MALDI-MS) sample loading were adopted directly from published data (17) without significant modification.

Fragmentation of Intact Apo A-IV

Intact apo A-IV was purified from normal serum by two-dimensional electrophoresis as described above. The protein was identified by Western blot using anti–apo A-IV antibodies (clone 6C2A7, Boehringer Mannheim). Fragmentation of apo A-IV was achieved by incubating purified apo A-IV with 0.15 M cyanogen bromide in formic acid 70%/acetonitrile 10%, which cleaves the protein at methionine residues in positions 30, 111, and 188 (18). Incubation was continued overnight in the dark at room temperature. The effectiveness of the digestion was confirmed by SDS electrophoresis in a discontinuous gradient system.

Commercially Available Apolipoprotein and Apolipoprotein Antibodies

As a preliminary control and confirmation of our procedures, we obtained purified apo E_3 and apo E_4 from a commercial source (Chemicon) and tested their antipermeability properties in the bioassay under the same conditions as our isolated proteins, *i.e.*, 1 μg of the apolipoprotein was added to 20 μl of the FSGS serum, which was then incubated with the glomeruli.

In other control experiments, monoclonal anti–apo A-IV and polyclonal anti–apo E and J antibodies, obtained from the commercial sources named above, were added separately to 1 μg of the corresponding purified apolipoprotein, mixed with 20 μl of the FSGS serum, and incubated with the glomeruli, as described above.

Finally, the same antibodies were preincubated in separate experiments with 20 μl of whole normal serum at 37°C for 10 min, and 10 μl were incubated with the FSGS serum and glomeruli, as described above.

Results

Isolation of Proteins with In Vitro Antipermeability Activity

Table 1 shows the effect of the FSGS sera on glomerular P_{alb} either alone or in combination with normal whole serum. Once having confirmed that P_{alb} values in healthy isolated glomeruli were normalized when the donor serum was added to the FSGS serum in the bioassay, we proceeded to isolate the inhibitory factors from normal serum following the multistep procedure described above. The proteins that resulted from each step of the procedure were tested for antipermeability activity by the bioassay. At the end of the chromatographic extraction procedure, the resulting proteins were separated further by two-dimensional electrophoresis under nonreducing conditions into approximately 50 spots or families of isoforms. Each spot was recovered from the gel and renatured, and its inhibitory potential on the permeability activity was evaluated by the bioassay. Table 1 also demonstrates the inhibitory effect of six spots on permeability activity; these spots are numbered 1 to 6 in Figure 1A.

Protein Identification

One of the antipermeability protein spots proved to be a polymer of at least five subunits of different molecular masses linked by disulfide bridges, whose characterization is currently in progress. The five remaining proteins with inhibitory activity had apparent molecular masses of 28, 36, 36, 42, and 80 kD (Figure 1A), which were comparable also under reducing conditions. All of these proteins were identified by MALDI-MS (Table 2). The 28-kD protein (spot 6) was a fragment of apo A-IV. The two isoforms weighing 36 kD (spots 4 and 5) were apo E_2 and apo E_4. The 42-kD protein (spot 3) was apo L, and the 80-kD protein (spot 1) was identified as high-molecular-weight apo J (19–21). Apo E_3 and the low-molecular-weight components of apo J, including NA 1 and NA 2, did not present antipermeability activity. The tryptic fragmentation pattern of the five proteins showing the putative amino acid sequence and the position of the peptide inside the sequence (22–25) is reported in Table 2. The tryptic composition of the purified apo A-IV fragment, also taking into account its molecular weight, had 53% homology with the theoretical sequence of the intact protein. The MALDI-MS pattern of the remaining four proteins reached a homology between 20 and 50%, indicating a clear identification of the proteins (18). Four of the apolipoproteins characterized by MALDI-MS were also identified by

Table 1. P_{alb} in isolated glomeruli induced by FSGS serum alone or in combination with normal serum or isolated proteins with or without corresponding antibodies[a]

	Protein Identification	P_{alb}
FSGS serum alone		0.95 ± 0.03
+ pooled normal serum		0.09 ± 0.01
+ spot 1	80 kD apo J	0.04 ± 0.01
+ spot 2	Polymer	0.05 ± 0.01
+ spot 3	Apo L	0.10 ± 0.02
+ spot 4	Apo E_2	0.01 ± 0.01
+ spot 5	Apo E_4	0.06 ± 0.06
+ spot 6	Apo A-IV fragment	0.05 ± 0.01

[a] P_{alb}, albumin permeability; FSGS, focal segmental glomerulosclerosis; apo, apolipoprotein.

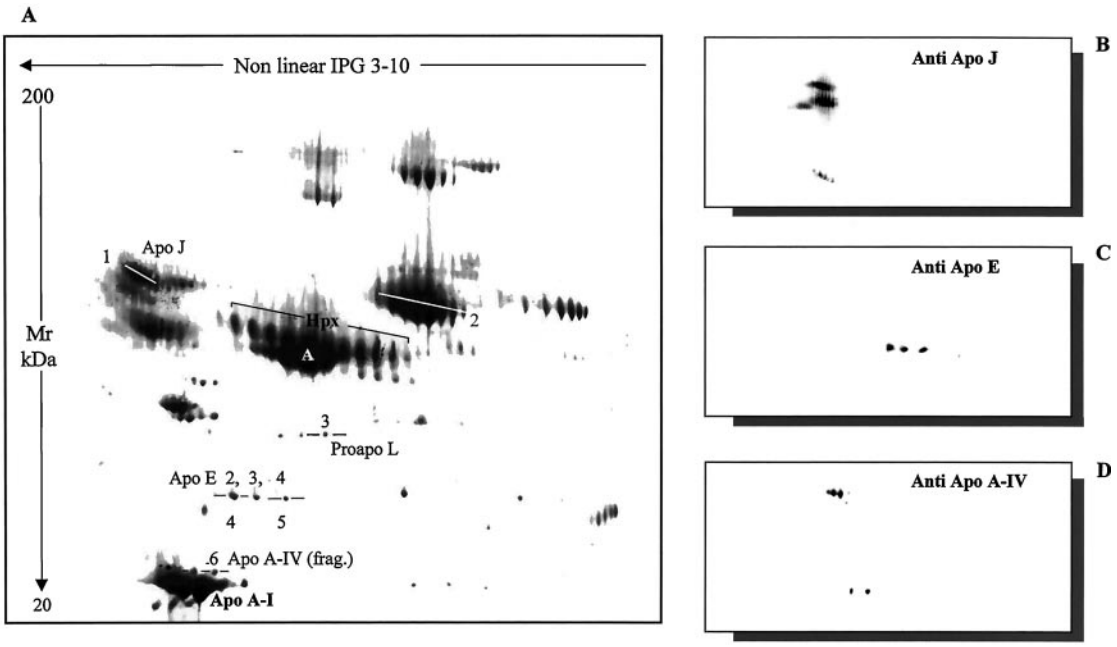

Figure 1. (A) Two-dimensional electrophoresis pattern of normal serum after the purification procedure described in the text. Proteins demonstrating antipermeability activity in the isolated glomeruli assay are numbered 1 to 6 (see also Table 1). A, albumin; Hpx, hemopexin; IPG, immobilized pH gradient; Mr, relative molecular weight. (B) Western blot analysis with polyclonal anti-human apolipoprotein J (apo J) antibodies. (C) Western blot analysis with polyclonal anti-human apo E antibodies. (D) Western blot analysis with monoclonal anti-human apo A-IV antibodies. Both intact and apo A-IV 28-kD fragment were recognized by the antibodies.

specific antibodies in the Western blot assay (Figure 1, B through D).

Effect of Commercial Grade Apo E and Apo J and Fragmented Apo A-IV

To demonstrate that the antipermeability effect of the apolipoproteins was not due to an artifact caused by the purification procedures, we repeated the experiment with commercial grade apo E_3 and E_4. Commercial E_4 blocked the permeability activity of the FSGS serum (P_{alb} = 0.00), whereas commercial E_3 did not (P_{alb} = 0.91). In a second approach, polyclonal antibodies against purified apo E and apo J were added to the bioassay (together with apo E or apo J), which abolished their antipermeability effect (Figures 2 and 3). The 28-kD fragment of apo A-IV inhibited permeability activity, whereas the intact apolipoprotein purified under the same conditions did not (Figure 4). To strengthen our impression that only fragments of the apo A-IV molecule block the permeability activity in the bioassay, cyanogen bromide was used to cleave the purified protein. The digestion yielded four fragments that, as shown in Figure 4, did indeed abrogate the permeability activity of the FSGS serum, whereas the intact protein did not.

Apolipoprotein Antibody Studies

In separate experiments, normal whole serum was preincubated with specific antibodies against apo J, apo E, and apo A-IV. Anti–apo J and anti–apo E antibodies prevented the inhibitory effect of normal serum on FSGS serum-induced permeability (P_{alb} = 0.93 and 0.69, respectively), whereas no effect was produced by the anti–apo A-IV antibodies (P_{alb} = 0.33; Figure 5).

Discussion

The present study identified from the serum of normal subjects six purified proteins that are capable of inhibiting glomerular P_{alb} induced by FSGS serum *in vitro*. One of the proteins is a polymer from which we are currently attempting to isolate the active subunit for further characterization. The other five proteins were identified by MALDI-MS and Western blot as apolipoprotein components of high-density lipoprotein (HDL; in order of decreasing molecular mass): 80 kD apo J, apo L, apo E_2, apo E_4, and a fragment of apo A-IV. Intact apo A-IV has a molecular mass of 46 kD (22), but several fragments that have a molecular mass and pI comparable to the 28-kD fragment described in the present study have been described in normal serum (26), which suggests that our finding was not an artifact of the isolation procedure. Also, apo J presented isoforms; only the high-molecular-weight components with 80 kD was protective, whereas the low-molecular-weight fractions did not play any effect.

We have tentatively concluded that the described apolipoproteins were responsible for the antipermeability effect of normal serum on the basis of the results obtained from the antibody studies. This does not exclude that other serum components may lose their activity during the preparative procedure. The following reasons point to a central role of apolipoproteins. In fact, depleting normal serum of apo E or apo J abrogated the inhibitory effect of normal serum and restored

Table 2. Tandem mass spectometric identification of apolipoproteins J, E_2, E_4, L and A–IV[a]

Protein	Peptide n°	Experimental [M + H]	Predicted [M + H^1]	Sequence	Position in Protein
Apo J (spot 1)	1	1074.60	1074.60	RPHFFFPK	215–222
	2	1392.66	1392.68	ASSIIDELFQDR	183–194
	3	1793.81	1799.80	FMETVAEKALQEYR	430–443
	4	1871.88	1781.84	QQTHMLDVMQDHFSR	168–182
	5	2313.25	2313.17	VTTVASHTSDSDVPSGVTEVVVK	386–408
Apo L (spot 3)	1	1274.60	1274.64	VNEPSILEMSR	306–316
	2	1595.72	1595.83	WWTQAQAHDLVIK	219–231
	3	1630.80	1630.79	VTEPISAESGEQVER	291–305
	4	2487.10	2487.25	EFLGENISNFLSLAGNTYQLTR	241–262
Apo E_2 (spot 4)	1	898.47	898.43	FWDYLR	33–38
	2	1646.78	1646.79	GEVQMLGQSTEELR	120–134
	3	1729.83	1729.84	SELEEQLTPVAEETR	76–90
	4	1752.90	1752.89	KVEQAVETEPEPELR	1–15
	5	2729.38	2729.39	WVQTLSEQVQEELLSSQVTQELR	39–61
Apo E_4 (spot 5)	1	898.47	898.43	FWDYLR	11–16
	2	1646.78	1646.79	GEVQAMLGQSTEELR	98–112
	3	1729.83	1729.84	SELEEQLTPVAEETR	54–68
	4	2729.38	2729.39	WVQTLSEQVQEELLSSQVTQELR	17–39
Apo A–IV (spot 6)	1	975.55	975.51	ISASAEELR	122–130
	2	1083.56	1083.54	LTPYADEFK	67–75
	3	1104.59	1104.57	LEPYADQLR	1–9
	4	1258.68	1258.68	IDQTVEELRR	78–87
	5	1258.68	1258.68	GNLKGNTEGLQK	142–153
	6	1287.62	1287.67	TQVNTQAEQLR	10–20
	7	1558.76	1558.80	LNHQLEGLTFQMK	100–112
	8	1933.97	1933.99	ENADSLQASLRPHADELK	36–53

The tryptic fragments here reported were obtained by the fragmentation of two proteins purified by two-dimensional electrophoresis that correspond to spots 1, 3, 4, 5, and 6 of Figure 1.

[a] Five tryptic peptides analyzed from spot 1 correspond to apo J with a 16% homology. Eight tryptic peptides analyzed from the 28-kD protein (spot 6) correspond to the sequence of human apo A–IV ((18)). Considering the lower molecolar mass and the homology of the tryptic composition (59%), the protein was considered a fragment of apo A–IV. Four tryptic peptides analyzed from the 42-kD digest correspond to apo L precursor ((19)). These peptides covered 17% of apo L precursor. For spot 4 and spot 5, five and four peptides correspond to E_2 and E_4 with a 41% homology.

the permeability activity of the FSGS serum. It is unclear why depleting one or the other of these apolipoproteins should block the entire effect of the normal serum, when the other apolipoproteins remain after the precipitation. It may be that the antipermeability effect depends on an unknown interaction among the various apolipoproteins. All of the antipermeability activity that we observed in the serum was associated with the hydrophobic rather than the hydrophilic protein fractions, and thus it is possible that purified lipids as well may exert glomerular antipermeability activity in relation to the FSGS serum. The purified apolipoproteins or the commercial apolipoproteins that we added to the FSGS serum should have resulted in the physiologic range (27,28), according to our calculations.

Perhaps apolipoproteins also play a role in the pathogenesis of FSGS or other glomerulopathies *in vivo*. FSGS serum may be deficient in factors such as particular apolipoproteins that normally bind and protect the glomerular basement membrane against the putative permeability factor or its effects. In fact, this theory suggests that neither the permeability factor nor the inhibitor need be substances exceedingly exotic or rare; the glomerular damage may result from an imbalance between the two. If not in the initiation of the disease, apolipoproteins may be involved in the progression of FSGS, as well as other forms of noninflammatory glomerulonephritis. Patients with the nephrotic syndrome demonstrate marked variations in the lipoprotein profile, usually with elevation of low-density and very-low-density lipoproteins and variable levels of HDL (29,30). HDL may be lost in the urine in patients with unselective proteinuria such as those with FSGS (29). Urinary loss of inhibitory substances may produce a new equilibrium between factors that increase and those that inhibit permeability activity. In conditions characterized by a high degree of selective proteinuria, such as minimal change nephropathy, apolipoprotein loss should not occur.

The literature regarding the role of apolipoprotein polymor-

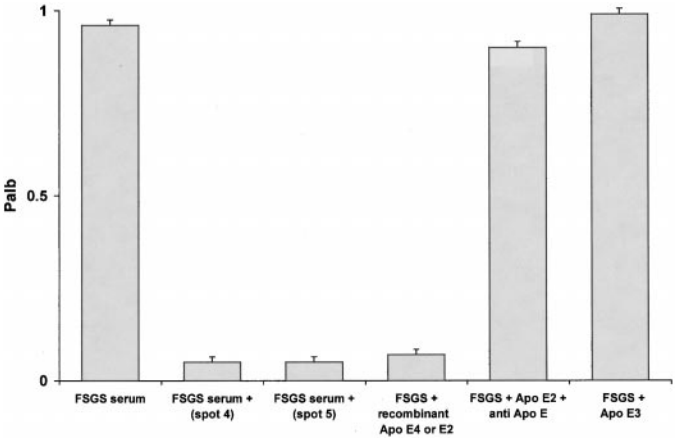

Figure 2. Albumin permeability activity (P_{alb}) of focal segmental glomerulosclerosis (FSGS) serum in the isolated glomeruli assay in combination with apo E_2 or apo E_4 purified by our procedure or of commercial source. Apo E_2 and E_4 prevents the permeability activity of FSGS serum, whereas the concomitant presence of anti–apo E antibodies restored it. Apo E_3 had no effect.

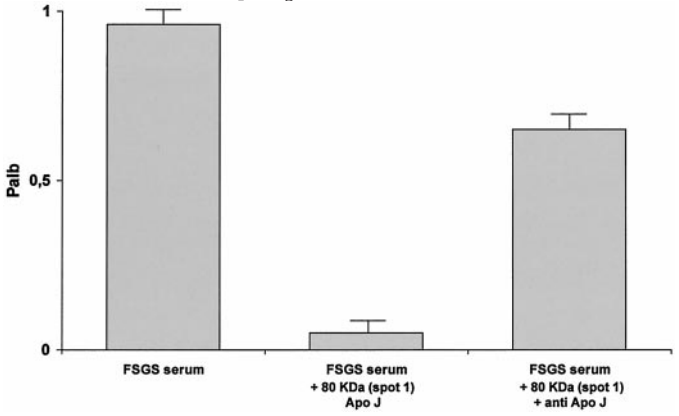

Figure 3. P_{alb} in the isolated glomeruli assay of FSGS serum alone or in combination with apo J purified with our procedure. Apo J abolished the permeability activity induced by FSGS serum, whereas the concomitant presence of anti–apo J antibodies restored it.

phisms in human disease is growing, particularly regarding apo A and E. Apo A-IV plasma levels have been correlated with an increase (31), decrease (32), or no change (33) in macrovascular atherosclerotic lesions. The effect of A-IV fragments on atherosclerotic lesions has not been studied. The precise relationship between atherosclerosis and FSGS is unknown, but analogous pathophysiologic mechanisms have been noted (34). The polymorphism of the apo E gene also influences plasma cholesterol and low-density lipoprotein concentrations. Apo E has been associated with reduced risk of nephropathy in patients with non–insulin-dependent diabetes mellitus (35), whereas some isoforms may be associated with lipoprotein glomerulopathy (36) and diabetic nephropathy in insulin-dependent patients (37). The amino acid sequence of apo J is virtually homologous with the cytolysis inhibitor SP-40,40 and may have an inhibitory effect on complement-mediated cell lysis (21). Finally, apo L has only recently been isolated in pancreatic exocrine cells and cloned by Duchateau *et al.* (23). The present article demonstrates that it is also readily extractable from normal serum.

We can only speculate regarding the mechanisms by which apolipoproteins protected the isolated glomeruli from the permeability activity of the FSGS serum in our study. Apolipoproteins are capable of binding to receptors, to heparin, to other lipoproteins, and so forth (38). They may also bind the putative permeability factor of FSGS. However, the phenomenon that we observed may be reserved to the *in vitro* environment. The isolated glomeruli used in the bioassay had been stripped of their capsules, which may expose the negative charges of the external vessels to factors in the medium that nonspecifically coat the glomeruli and prevent interactions with the permeabil-

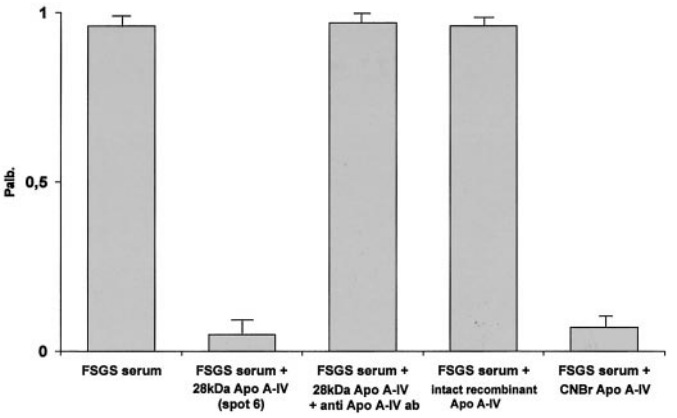

Figure 4. P_{alb} in the isolated glomeruli assay of FSGS serum alone and in combination with the 28-kD fragment of apo A-IV, intact apo A-IV, or apo A-IV fragmented with cyanogen bromide (CNBr). The 28-kD fragment of apo A-IV blocked the FSGS permeability activity as did apo A-IV fragmented with CNBr. Intact apo A-IV had no effect. Anti–apo A-IV antibodies restored the permeability effect of FSGS serum when added to the purified fragment.

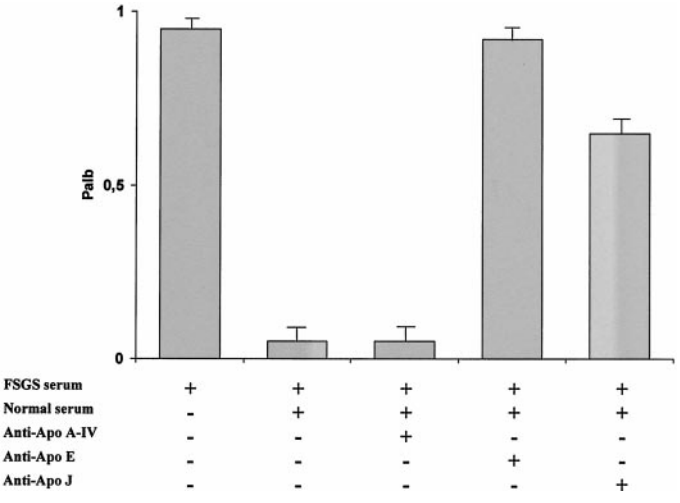

Figure 5. P_{alb} of FSGS serum alone or in combination with normal serum. Pretreatment of the normal serum with anti–apo E or anti–apo J antibodies blocked its inhibitory effect, and the permeability activity of FSGS serum was restored. Anti–apo A-IV had no effect.

ity factor. Finally, the antipermeability effect of the apolipoproteins may not be specific to FSGS; the pathogenetic mechanisms of other glomerulopathies, which may not involve a permeability factor, may respond to apolipoproteins as well.

In conclusion, apolipoproteins purified from normal serum may prevent glomerular permeability alterations induced by FSGS serum *in vitro*. In a disease such as FSGS, in which few certainties exist regarding the pathogenesis and treatment, this observation merits further confirmation and explanation.

Acknowledgments

We gratefully acknowledge the continued interest and financial support of the Fondo Malattie Renali del Bambino. We thank Dr. Moreno Pagnanelli (Amersham Pharmacia Biotech) for his technical support. MALDI-MS was performed in the laboratory of protein analysis of WITA GmbH (Teltow, Germany). We also thank Dr. Marco Baralle for furnishing the monoclonal anti–apo A-IV antibodies. Dr. Andres Muro kindly read the manuscript and provided astute suggestions. This work was also supported by a grant from the Italian Ministry of Health to G. Gaslini Institute.

References

1. Dall'Amico R, Ghiggeri GM, Carraro M, Artero M, Ghio L, Zamorani E, Zennaro C, Basiel G, Montini G, Rivabella L, Cardillo M, Scalamogna M, Ginevri F: Prediction and treatment of focal segmental glomerulosclerosis after renal transplantation in children. *Am J Kidney Dis* 14: 1048–1058, 1999
2. Artero ML, Sharma R, Savin VJ, Vincenti F: Plasmapheresis reduces proteinuria and serum capacity to injure glomeruli in patients with recurrent focal glomerulosclerosis. *Am J Kidney Dis* 23: 574–581, 1994
3. Andresdottir MB, Ajubi N, Croockewit S, Assmann KJM, Hilbrands LB, Wetzels JFM: Recurrent focal glomerulosclerosis: Natural course and treatment with plasma exchange. *Nephrol Dial Transplant* 14: 2650–2656, 1999
4. Dantal J, Bigot E, Bogers W, Testa A, Kriaa F, Jacques Y, Hurault-de-Ligny B, Niaudet P, Charpentier B, Soulillou JP: Effect of plasma protein adsorption on protein excretion in kidney transplant recipients with recurrent nephrotic syndrome. *N Engl J Med* 330: 7–14, 1994
5. Savin VJ, Sharma R, Sharma M, McCarthy ET, Swan SK, Ellis E, Lovell H, Warady B, Gunwar S, Chonko AM, Artero M, Vincenti F: Circulating factor associated with increased glomerular permeability to albumin in recurrent focal segmental glomerulosclerosis. *N Engl J Med* 334: 878–883, 1996
6. Godfrin Y, Dantal J, Bouhours JF, Heslan JM, Soulillou JP: A new method of measuring albumin permeability in isolated glomeruli. *Kidney Int* 50: 1352–1357, 1996
7. Dantal J, Godfrin Y, Koll R, Perretto S, Naulet J, Bouhours JF, Soulillou JP: Antihuman immunoglobulin affinity immunoadsorption strongly decreases proteinuria in patients with relapsing nephrotic syndrome. *J Am Soc Nephrol* 9: 1709–1715, 1998
8. Sharma M, Sharma R, McCarthy ET, Savin VJ: "The FSGS factor": Enrichment and in vivo effect of activity from focal segmental glomerulosclerosis plasma. *J Am Soc Nephrol* 10: 552–561, 1999
9. Savin VJ, Sharma R, Artero M, Vincenti F: Normal serum prevents the increase in glomerular permeability caused by sera from patients with focal glomerular sclerosis (FGS) [Abstract]. Presented at the XXXIst Congress of the European Renal Association/European Dialysis and Transplant Association, July 3–6, 1994, Vienna, p 103
10. Savin VJ, Sharma R, Lovell HV, Welling DJ: Measurement of albumin reflection coefficient using isolated rat glomeruli. *J Am Soc Nephrol* 3: 1260–1269, 1992
11. Nelson E, Eyatan E, Notsani BE, Sigrist H, Sigrist-Nelson K, Citler C: Isolation of a chloroplast N_1N^1–dicycloherylcarbodiinide–binding proteolipid, active in proton translocation. *Proc Natl Acad Sci USA* 74: 2375–2378, 1997
12. Bjellqvist B, Pasquali C, Ravier F, Sanchez JC, Hochstrasser D: A non-linear wide-range immobilized pH gradient for two-dimensional electrophoresis and its identification in a relevant pH scale. *Electrophoresis* 14: 1357–1365, 1993
13. Musante L, Candiano G, Ghiggeri GM: Resolution of fibronectin and other uncharacterized proteins by two dimensional polyacrylamide electrophoresis with thiourea. *J Chromatogr* 705: 351–356, 1998
14. Candiano G, Porotto M, Lanciotti M, Ghiggeri GM: Negative staining of proteins in polyacrylamide gels with methyl trichloroacetate. *Anal Biochem* 243: 245–248, 1998
15. Lee C, Levin A, Branton D: Copper staining: A five minute protein stain for sodium dodecyl sulfate-polyacrylamide gels. *Anal Biochem* 166: 308–312, 1997
16. Hager DA, Burgess RA: Elution of proteins from sodium dodecyl sulfate and renaturation of enzymatic activity: Results with Sigma subunit of *Escherichia coli* RNA polymerase, wheat germ DNA topoisomerase and other enzymes. *Anal Biochem* 109: 76–87, 1980
17. Iarsson T, Norbeck J, Karlsson M, Karlsson KA, Blomberg A: Identification of two dimensional gel electrophoresis resolved yeast proteins by matrix-assisted laser desorption ionization mass spectrometry. *Electrophoresis* 18: 418–423, 1997
18. James RW, Hochstrasser D, Tissot JD, Funk M, Appel R, Barja F, Pellegrini C, Muller AF, Pometta D: Protein heterogeneity of lipoprotein particles containing apolipoprotein A-I without apolipoprotein A-II and apolipoprotein A-I with apolipoprotein A-II isolated from human plasma. *J Lipid Res* 29: 1557–1571, 1988
19. Hochstrasser AC, James RW, Martin BM, Harrington M, Hochstrasser D, Pometta D, Merril CR: HDL particle associated proteins in plasma and cerebrospinal fluid: Identification and partial sequencing. *Appl Theor Electrophor* 1: 73–76, 1988
20. Ghiso J, Matsubara E, Koudinov A, Choi-Miura NH, Tomita M, Wisniewski T, Frangione B: The cerebrospinal-fluid soluble form of Alzheimer's amyloid beta is complexed to SP-40,40 (apolipoprotein J), an inhibitor of the complement membrane-attack complex. *Biochem J* 293: 27–30, 1993
21. James RW, Hochstrasser AC, Borghini I, Martin B, Pometta D, Hochstrasser D: Characterization of a human high density lipoprotein-associated protein, NA1/NA2: Identity with SP-40,40, an inhibitor of complement-mediated cytolysis. *Arterioscler Thromb* 11: 645–652, 1991
22. Karathanasis SK: Apolipoprotein multigene family: Tandem organization of human apolipoprotein A-I, C-III and A-IV genes. *Proc Natl Acad Sci USA* 82: 6374–6378, 1995
23. Duchateau PN, Pullinger CR, Orellana RE, Kunitake ST, Naya-Vigen J, O'Connor PM, Malloy MJ, Kane JP: Apolipoprotein L, a new high density lipoprotein apolipoprotein expressed by the pancreas. *J Biol Chem* 272: 25576–25582, 1997
24. Kirszbaum L, Sharpe JA, Murphy B, D'Apice AJ, Classon B, Hudson P, Walker ID: Molecular cloning and characterization of the novel, human complement-associated protein, SP-40,40: A

link between the complement and reproductive systems. *EMBO J* 8: 711–718, 1989
25. Das HK, McPherson J, Bruns GA, Karathanosls SK, Breslow JC: Isolation, characterization, and mapping to chromosome 19 of the human apolipoprotein E gene. *J Biol Chem* 260: 6240–6247, 1985
26. Hochstrasser DF, Tissot J: Clinical application of high-resolution two-dimensional polyacrylamide gel electrophoresis. In: Advances in Electrophoresis, edited by Chrambach A, Dum MJ, Rodole BJ, Weinheim, Weinheim, Germany, VCH Editions, 1992, pp 267–376
27. Ghiselli G, Krishnan S, Beigel J, Gotto A: Plasma metabolism of apolipoprotein A-IV in humans. *J Lipid Res* 27: 813–827, 1986
28. Kushwa RS, Hazzard WR, Wahl PW, Hoover JJ: Type III hyperlipoproteinemia: Diagnosis in whole plasma by apolipoprotein E immunoassay. *Ann Intern Med* 87: 509–516, 1977
29. Short CD, Durrington PN, Mallick NP, Hunt LP, Tetlow L, Ishola M: Serum and urinary high density lipoprotein in glomerular disease with proteinuria. *Kidney Int* 29: 1224–1228, 1986
30. Van Gor H, van der Horst M, Atmosoerodjo J, Joles JA, vanTol A, Grond J: Renal apolipoprotein in nephrotic rats. *Am J Pathol* 142: 1804–1812, 1993
31. Verges BL, Lagrost L, Vaillant G, Petit JM, Cohen M, Gambert P, Brun JM: Macrovascular disease is associated with increased plasma apolipoprotein A-IV levels in NIDDM. *Diabetes* 46: 125–132, 1997
32. Duverger N, Tremp G, Caillaud JM, Emmanuel F, Castro G, Fruchart JC, Steinmetz A, Denefle P: Protection against atherogenesis in mice mediated by human apolipoprotein A-IV. *Science* 273: 966–968, 1996
33. Bai H, Saku K, Liu R, Oribe Y, Yamamoto K, Arakawa K: Polymorphism of the apolipoprotein A-IV gene and its significance in lipid metabolism and coronary heart disease in a Japanese population. *Eur J Clin Invest* 26: 1115–1124, 1996
34. Diamond JR, Karnovsky MJ: Focal and segmental glomerulosclerosis: Analogies to atherosclerosis. *Kidney Int* 33: 917–924, 1988
35. Kimura H, Suzuki Y, Gejyo F, Karasawa R, Miyazaki R, Suzuki S, Arakawa M: Apolipoprotein E4 reduces risk of diabetic nephropathy in patients with NIDDM. *Am J Kidney Dis* 31: 666–673, 1998
36. Oikawa S, Suzuki N, Sakuma E, Saito T, Namai K, Kotake H, Fujii Y, Toyota T: Abnormal lipoprotein and apolipoprotein pattern in lipoprotein glomerulopathy. *Am J Kidney Dis* 18: 553–558, 1991
37. Werle E, Fiehn W, Hasslacher C: Apolipoprotein E polymorphism and renal function in German type 1 and type 2 diabetic patients. *Diabetes Care* 21: 994–998, 1998
38. Mahley RW: Apolipoprotein E: Cholesterol transport protein with expanding role in cell biology. *Science* 240: 622–630, 1988