The Focal Segmental Glomerulosclerosis Permeability Factor: Biochemical Characteristics and Biological Effects

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Focal segmental glomerulosclerosis (FSGS) is characterized by steroid resistant nephrotic syndrome and progression to end-stage renal disease. Proteinuria in certain patients with FSGS may be caused by a circulating factor (FSGS permeability factor [FSPF]). The current report documents the biochemical characteristics and the biological and molecular effects of 70% ammonium sulfate supernatant of plasma from patients with recurrence of FSGS after transplantation (FSGS 70% supernatant). FS permeability activity, defined as the capacity of plasma from patients with FSGS to increase albumin permeability (P_{alb}) of isolated glomeruli, was assessed *in vitro*. Permeability activity was not affected by lyophilization. FSPF bound strongly to matrices containing Mono-Q anion exchanger or protein A. It eluted from matrix-bound Cibacron blue F3GA over a wide range of salt concentrations, indicating a potential binding with other proteins, such as albumin. FSPF caused a maximal increase in P_{alb} within 2 mins of incubation *in vitro*. Cellular proteins isolated from glomeruli with increased P_{alb} showed decreased tyrosine phosphorylation of focal adhesion kinase, paxillin, and other proteins. Tyrosine phosphatase inhibition prevented the increase in P_{alb}. Intravenous administration of as little as 3 mg protein in FSGS 70% supernatant increased P_{alb}, while 9 mg or more were required to produce proteinuria. We conclude that FSPF is a low-molecular-weight protein, carries an anionic charge, and binds to protein A. Effects of FSPF on the glomerular permeability barrier are rapid and dose dependent and involve signaling through altered phosphorylation of cellular proteins. Identification of these biochemical and biological characteristics may be used to design strategies for removing FSPF from circulation and for purification and identification of this factor. Exp Biol Med 229:85–98, 2004

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Focal segmental glomerulosclerosis (FSGS) is characterized by nephrotic range proteinuria and a high incidence of progression to end-stage renal disease. FSGS affects both adult and pediatric populations. Diagnosis of FSGS is made by histopathological analysis of renal tissue (1). Recent reports suggest a several-fold increase in the incidence of FSGS during the last two decades (2–4). Reasons for this increase are not clear. Putative risk factors for FSGS include racial background (5–7) and family history (8, 9). Several genetic abnormalities involving proteins of the glomerular epithelial cell cytoskeleton or of cell junction proteins have been described in patients with familial nephrotic syndrome and FSGS (10–16). Abnormalities in expression of these proteins are also found in experimental models of progressive glomerular injury (17, 18). Responses to viral infections or to toxins have also been implicated, especially in severe forms or in collapsing FSGS (19, 20). The etiology of idiopathic FSGS remains unknown.

A circulating substance is believed to cause FSGS in some patients. Rationale for this interpretation include the following observations. First, FSGS recurs after renal transplantation in 30%–40% of patients in whom FSGS is the primary renal disease (21–23). Recurrence of proteinuria can be observed almost immediately after transplantation or may be delayed for days or weeks. Recurrent disease often results in the loss of function of the allograft (21). Second, plasmapheresis during early stages of the disease or immediately after transplantation may reduce proteinuria and stabilize renal function (24–28). Immunoadsorption of plasma proteins has also been found to be effective in some patients (29, 30). Third, injection of plasma or fractions of plasma from patients with recurrent FSGS may cause proteinuria or albuminuria in experimental animals (31). Finally, FSGS serum, plasma, or discarded plasmapheresis fluid (PPF) can increase glomerular albumin permeability

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(P_{alb}) in an *in vitro* functional assay (32), and infusion of patient material results in proteinuria and albuminuria (33, 34). We have demonstrated that FS permeability activity, defined by an increase in P_{alb} caused by the focal segmental glomerulosclerosis permeability factor (FSPF), can be neutralized by normal plasma (35). Certain plasma proteins, such as apolipoproteins, can block or neutralize its activity (36). These results support the hypothesis that circulating FSPF(s) may be responsible for glomerular damage and for initiation of glomerular events that contribute to the development of proteinuria in FSGS.

We have reported a protocol of sequential precipitation of plasma of patients with recurrent FSGS. This protocol results in a 100-fold enrichment of FS permeability activity. Material derived as 70% supernatant increases P_{alb} of isolated glomeruli and carries all the activity of the starting material (33). We have also reported that intravenous injection of the 70% supernatant causes proteinuria and albuminuria in rats and that P_{alb} of glomeruli isolated from these proteinuric rats exhibit increased P_{alb} without further experimental treatment (34). Neither the amount of 70% supernatant that must be injected to increase P_{alb} nor the amount required to cause proteinuria has been defined. The cellular mechanisms that are responsible for increased P_{alb} are also unknown. The physicochemical characteristics of FSPF have not been defined, although understanding these characteristics may be essential to the development of therapeutic strategies in FSGS. The current studies were designed to address these aspects of the chemistry and biological activity of FSPF.

In the present report, we describe certain characteristics of the active component as evidenced by binding to chromatographic materials. We further characterize the *in vivo* effect of FSPF on P_{alb}, the time course of the *in vitro* effects, changes in tyrosine phosphorylation of glomerular cellular proteins, and effects of blocking these changes in tyrosine phosphorylation. This information will be useful in understanding the mechanisms that lead to proteinuria in FSGS and will permit development of methodology for purification and characterization of FSPF and for its specific removal from affected patients.

Materials and Methods

Chemicals and Supplies. Anion exchanger Mono-Q, cation exchanger Mono-S, agarose-bound protein A, and Coomassie Blue Reagent for protein assay were obtained from Bio-Rad Laboratories (Hercules, CA). Silica-bound protein A was obtained from Prosorba, Imré Corporation (Seattle, WA). Concanavalin A–agarose (con A–agarose), agarose-bound heparin, Cibacron blue F3GA affinity blue gel (blue gel), DEAE-Sephacel, bovine serum albumin (BSA), sodium orthovanadate, HPLC, and analytical grade chemicals for buffers and other reagents were obtained from Sigma Chemical Company (St. Louis, MO). Spectra-Por dialysis tubing was obtained from Spectrum Medical Industries Inc. (Laguna Hills, CA). Deionized water was purified using NANO-Pure Ultrapure Water System from Barnstead (Dubuque, IA). Precast 4%–20% gradient mini-gels from Novell Experimental Technology Novex (Invitrogen, San Diego, CA) and equipment from Hoefer Scientific Instruments (San Francisco, CA) were used for electrophoresis. Protein G–sepharose was purchased from Sigma Chemical. Mouse anti-FAK monoclonal IgG_1 (Upstate Biotechnology, Lake Placid, NY), mouse antipaxillin monoclonal antibody, mouse antiphosphotyrosine monoclonal antibody (Transduction Laboratories, Lexington, KY), mouse antiactin IgM (Calbiochem, San Diego, CA), and HRP-conjugated rabbit anti-mouse IgG (Sigma Chemical), HRP-conjugated sheep anti-mouse IgG (Calbiochem), HRP-conjugated goat anti-mouse IgM (Calbiochem), ECL reagent (Amersham-Pharmacia Biotech, Piscataway, NJ), Hybond-ECL nitrocellulose membrane (Amersham-Pharmacia), and Biomax-MR photographic film (Kodak, Rochester, NY) were used for Western blotting.

Source of FSGS and Normal Plasma. Serum or plasma specimens were obtained from patients in whom the diagnosis of FSGS in native kidneys or in transplanted kidneys had been made. Details of the criteria used for the diagnosis of FSGS have been described earlier (24, 32, 33). In the studies described here, serum samples and plasmapheresis fluid (PPF), obtained during the course of therapeutic plasmapheresis from 12 patients, were tested. The decision to perform plasmapheresis was made by the primary physician in each case. The 12 patients included 10 adults and 2 children. Of these, 6 were male and 6 female. A number of these patients were represented in prior reports (33, 34), while others had not been studied in the past. Their demographic characteristics were comparable to those of patients previously reported (33). Each was on immunosuppressive medications as part of post-transplant therapy. Detailed information regarding their renal function and medications at the time of plasmapheresis was not available to us. Additional PPF was obtained from a male patient who experienced a recurrence of FSGS and allograft loss in 1985 and who has since been treated with thrice-weekly hemodialysis. This patient's serum, plasma, and plasmapheresis fluid have consistently shown high levels of FS activity *in vitro*. This patient was not nephrotic and had normal serum proteins; he was not on any immunosuppressive medications. Plasmapheresis was performed using citrate anticoagulation, and plasma removed was replaced with 0.9% sodium chloride and 5% human albumin solution. Discarded PPF was shipped to our laboratory on dry ice and was stored at −20°C.

Normal serum from healthy volunteers and discarded normal plasma from the Blood Center of Southeastern Wisconsin (Milwaukee, WI) were obtained. Each sample was stored at −20°C until use. Plasma from 35 normal individuals was pooled and designated normal pooled plasma (NPP). NPP was used to provide control material for activity studies and for protein purification.

Enrichment of FSPF from PPF and Identical Fractions from NPP. As described previously, an enriched preparation of FSPF (FSGS 70% supernatant) was obtained by removing inactive plasma components from the PPF (33). Briefly, cryoprecipitate was removed by centrifugation. Lipid-rich lipoproteins and chylomicrons were precipitated by dextran sulfate and calcium chloride and removed by centrifugation. From the post-lipoprotein supernatant, immunoglobulins and albumin-rich fractions were salted out at 50% and 70% ammonium sulfate saturation, respectively. Precipitates were not tested for activity. The supernatant at 70% ammonium sulfate saturation contained FS activity. FS activity was not present in other fractions (33). NPP was treated identically to obtain the supernatant at 70% ammonium sulfate. The same amount of total protein from FSGS or NPP 70% supernatant was used in studies of P_{alb} and in injection studies.

Experimental Animals and Preparation of Glomeruli. Animals were cared for according to NIH guidelines. All protocols were reviewed and approved by the Institutional Committee for care and use of animals at the Medical College of Wisconsin (MCW). Rats were maintained at the Animal Resource Center with free access to rat chow and drinking water.

For studies of P_{alb}, glomeruli were isolated from kidneys of normal male Sprague-Dawley rats (175–200 g). Rats were anesthetized with Metofane vapor (methoxyflurane, Mallinckrodt Veterinary Inc., Mundelein, IL), kidneys removed, and rats euthanized by ex-sanguination. Renal medulla was removed, and fine pieces of the cortical slices were passed through stainless steel sieves of decreasing pore sizes. Glomeruli were collected from atop the 200-mesh sieve. Details of the techniques and conditions used have been described (24, 32–35).

Collection of Urine and Determination of Protein and Creatinine. Pretreatment urine was collected from rats in metabolic cages. For intravenous injection of FSPF, rats (250–275 g) were anesthetized with intraperitoneal Brevital (methohexital sodium) at 50 mg/kg (Eli Lilly and Co., Indianapolis, IN). After injection of FSPF, rats were allowed to recover and placed in metabolic cages for urine collection (24 hrs).

Every fraction of plasma to be tested for FS activity was dialyzed in TBS (10 mM Tris-HCl containing 1% sodium chloride, pH 7.8). Protein concentration in urine or chromatography fractions was determined by Bradford's method using Coomassie Blue Dye Reagent (Bio-Rad) and spectrophotometric measurement of absorbance at 595 nm (37). Urinary creatinine was determined using a reagent kit from Sigma Chemical.

Detection of FS Activity in FSPF by *In Vitro* Functional Assay. We have defined FS activity as the ability of serum, plasma, or plasma fractions to increase albumin permeability (P_{alb}) after *in vitro* incubations (33). Fractions obtained during purification were tested for their capacity to increase glomerular P_{alb} using methods established in our laboratory. Details of the rationale and methodology for using isolated glomeruli and measurement of the reflection coefficient (σ_{alb}) and P_{alb} and its usefulness in the purification of FSPF have been described previously (33, 34, 38). In short, glomeruli from superficial cortex were isolated at room temperature by sieving in isolation/incubation medium (pH 7.4) containing 4% BSA as an oncotic agent. Extensively dialyzed and diluted normal and FSGS plasma fractions (20 μl aliquot) in TBS were incubated with glomeruli in a final volume of 1 ml at 37°C for 2–10 min. Incubated glomeruli were observed using videomicroscopy before and 1 min after exposure to a transcapillary oncotic gradient. The oncotic gradient resulted in influx of fluid and expansion of glomerular capillaries. The relative volume increase (ΔV) of each glomerulus was used to calculate albumin convectional permeability (P_{alb}). P_{alb} is a dimensionless variable with a minimum of zero in normal glomeruli and a maximum of 1.0 after injury to the permeability barrier.

For studies of proteinuria in rats after injection of FSPF, we have reported numerical values. For studies of binding and recovery of FSPF from various chromatography media, we have defined $P_{alb} \geq 0.5$ as positive. Lower P_{alb} activity in patient sera may be present, but we have selected 0.5 as a cutoff because it exceeds the average value derived using sera of normal people or patients without FSGS by 2 standard deviations and permits us to exclude nonspecific activity with assurance.

Incubation of glomeruli with medium containing pooled normal serum (1:50 dilution) served as a negative control, while incubation with known active FSGS plasma (1:50 dilution) served as a positive control (33).

Effect of Lyophilization and Reconstitution of Sera on FS Activity. Aliquots (50 μl) of serum from each of the 12 patients with recurrent FSGS were frozen at −20°C overnight. These samples then were further cooled on dry ice for 3 hrs and lyophilized. The dry residue was left at room temperature (about 21°C) for 3 days and reconstituted to the original volume with water. Then aliquots (20 μl) of fresh or reconstituted serum were used to determine P_{alb} in a final volume of 1 ml (1:50 dilution). FS activity of lyophilized specimens was compared to activity of serum that had not been lyophilized (20 μl in 1 ml, 1:50 dilution).

Removal of FSPF Using Chromatography Media. The FSGS 70% supernatant was used to test the binding of FSPF with each chromatography material packed in a gravity-flow column. Established principles and techniques of chromatography were followed for each analysis. We tested Cibacron blue F3GA gel (39, 40), con-A (41), heparin-agarose (42), and silica- and agarose-bound protein A (43) for affinity binding; and Mono-S (44), DEAE-Sephacel (45), and Mono-Q (46) for ionic exchange binding. Table 1 provides an outline of the chromatography materials, equilibration and sample loading buffers, and elution conditions employed for each chromatographic procedure. The FSGS 70% supernatant (10 mg total protein)

Table 1. Summary of Chromatography Materials and Conditions

No.	Chromatography material	Equilibration and loading conditions	Wash buffer	Elution conditions
1.	Cibacron blue F3GA affinity gel	0.025 M sodium phosphate, pH 7.8 (Buffer 1)	Buffer 1	0.1–1.0 M sodium chloride in Buffer 1
2.	Con A–sepharose	Kreb-Ringer-bicarbonate (Buffer 2)	Buffer 2	0.5 M α-methyl-D-manno-pyranoside in Buffer 2
3.	Heparin-agarose	0.02 M Tris-HCl pH 7.8 (Buffer 3)	Buffer 3	0.01–1 M potassium chloride in Buffer 3
4.	Protein A–agarose	0.025 M citrate pH 7.6 (Buffer 4)	Buffer 4	0.025 M citrate pH 2.6; eluate collected in 1 M Tris pH 7.8; second elution in 1 M sodium thiocyanate
5.	Mono-S cation exchanger	0.05 M MOPS buffer pH 7.4 (Buffer 5)	Buffer 5	0.1–1 M sodium chloride in Buffer 5
6.	DEAE-Sephacel	10 mM Bis-Tris, pH 6.5 (Buffer 6)	Buffer 6	0.01–1.0 M sodium chloride in Buffer 6
7.	Mono-Q anion exchanger	0.02 M Bis-Tris pH 6.0 (Buffer 7)	Buffer 7	0.5 M sodium chloride in Buffer 7

was dialyzed in the equilibration buffer for each chromatography technique. Each chromatography material was equilibrated in the buffer listed under "Equilibration and Loading Conditions" (Table 1). The FSGS 70% supernatant was chromatographed on each column, and the unbound fractions were collected. Each column was washed three times with equilibration buffer to remove the unbound fraction. Bound proteins were eluted using buffers described under "Elution Conditions" (Table 1). All fractions were dialyzed against TBS and concentrated to 1 ml, and 20-μl aliquots were used for P_{alb} assay. Binding of FSPF with a particular chromatography material was further confirmed using 70% supernatant preparations from several FSGS patients.

Effects of Injection of Different Amounts of FSPF. For studies of the effects of FSPF on renal and glomerular function *in vivo*, basal levels of urine protein were determined from a 24-hr urine sample prior to injection of FSPF. Seventy percent supernatant was prepared from FSGS plasma or NPP, dialyzed against saline, diluted to the experimentally designated protein concentration, sterilized by filtration through a membrane (0.2-μm pore size), and administered intravenously in a volume of 1 ml (0.5 ml/min) to rats as described. Total protein injected in these studies was 1, 3, 6, 9, and 12 mg/rat (approximately 4, 12, 24, 36, or 48 mg/kg body weight, respectively). Rats were allowed to recover from anesthesia and housed in metabolic cages for collection of urine for 24 hrs. In some rats, P_{alb} was determined at 3 hrs after injecting FSPF (12 mg/rat). At the end of study, rats were euthanized under anesthesia and kidneys retrieved for glomerular isolation. Urinary protein and creatinine were determined as described.

Time Course of Increase in P_{alb} *In Vitro*. To determine the earliest time when increased P_{alb} could be measured, glomeruli were isolated in 5% BSA medium as described previously. An aliquot of glomerular suspension was added to the observation chamber, and glomeruli were allowed to settle and adhere to the polylysine-coated glass surface. Adherent glomeruli were observed after the addition of medium containing FSPF (20 μg protein/ml) for 2–10 mins. Hypo-oncotic medium was then added and P_{alb} measured using changes in glomerular diameter that occurred within 1 min (38). In a limited number of studies, isolated glomeruli were allowed to equilibrate with isoncotic high-molecular-weight dextran and were observed after the addition of FSPF. In these studies, fluid was lost from glomerular capillaries, and glomerular diameter diminished when P_{alb} increased. The finding that isoncotic dextran caused capillary collapse provided definitive evidence that the capillary reflection coefficient for albumin decreased and that an increase in P_{alb} rather than any other change in glomerular characteristics was responsible for the experimental responses we observed (38).

***In Vitro* Change in Glomerular Protein Tyrosine Phosphorylation.** The rapid increase in P_{alb} is consistent with a signaling pathway such as protein phosphorylation. Since tyrosine phosphorylation of cytoskeleton-associated proteins focal adhesion kinase (FAK) and paxillin alters adhesion of cells to substrate, we postulated that the change in P_{alb} might be associated with changes in tyrosine phosphorylation of these proteins.

Glomeruli were incubated with FSPF for 3 mins in RPMI-1640, followed by homogenization in a lysis buffer (150 mM sodium chloride, 1.5 mM magnesium chloride, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM PMSF, and 200 μM sodium orthovanadate in 50 mM HEPES, pH 7.4). Protein was determined by modified Lowry's method using a Bio-Rad reagent kit (47).

Equal amounts of protein from all samples were loaded (1:1) in a sample buffer (4% SDS, 20% glycerol, 0.005%

bromophenol blue, 5% mercaptoethanol, 125 mM Tris-HCl, pH 6.8) and resolved by SDS-PAGE on a Tris-glycine (1-mm–thick minigel) at 4°C. Proteins were electrotransferred to nitrocellulose membrane (30 V, overnight at 4°C) using a transfer buffer (0.3% Tris base, 1.44% glycine, 20% methanol). Blots were incubated with 5% nonfat milk in TTBS (10 mM Tris base, 100 mM sodium chloride, 0.05% Tween-20, pH 9.7) and washed with TTBS as needed. Tyrosine phosphorylation of glomerular proteins was determined by Western blotting using a mouse antiphosphotyrosine monoclonal antibody (1:2000 in 5% milk) as the primary antibody (1 hr, room temperature, 3 × 10-min washes with TTBS) and an HRP-conjugated rabbit anti-mouse IgG (1:50,000 in 5% fat-free milk powder) as the secondary antibody (1 hr, room temperature, 3 × 10-min washes with TTBS). Images of the HRP-conjugated protein-antibody complexes were obtained by incubation with ECL reagent (Amersham-Pharmacia) followed by exposure to photography film.

Glomerular lysate was subjected to immunoprecipitation, SDS-PAGE, and immunoblotting to analyze the effect of FSPF on tyrosine phosphorylation of glomerular FAK and paxillin. Glomerular lysate (1 mg protein) was mixed with anti-FAK (10 μg/mg protein) or antipaxillin (10 μg/mg protein) antibody and incubated overnight at 4°C on a low-speed gyratory shaker. The immune complex was captured mixing with 100 μl of protein G agarose bead slurry for 2 hrs at 4°C. The beads were centrifuged (14,000 *g*, 1 min), washed with ice-cold lysis buffer (3×), mixed with the sample buffer (50 μl), and heated at 95°C for 5 mins, and the immunocomplex in the supernatant was used for SDS-PAGE (4%–20% gradient for FAK and a 12% gel for paxillin). Transfer of proteins from the gel to a nitrocellulose membrane, blocking, and washes were carried out as outlined previously. Mouse anti-antiphosphotyrosine antibody and HRP-conjugated rabbit anti-mouse IgG were used as the primary and secondary antibodies, respectively, to carry out Western blotting as described previously. Chemiluminescence reaction and detection of immune complexes were carried out following the manufacturer's instructions (Amersham-Pharmacia). Immunoblotting of actin in the glomerular lysate was carried out using mouse anti-actin antibody as the primary and goat anti-mouse IgM (Calbiochem) as the secondary antibody, respectively (48).

Effect of Inhibitor of Tyrosine Phosphatase on Altered P_{alb}. Glomeruli were isolated in 4% BSA medium. Glomeruli were incubated under standard conditions with FSGS 70% supernatant alone or with FSGS 70% supernatant in the presence of sodium orthovanadate (200 μM), a phosphatase inhibitor. P_{alb} was measured after 10 mins of incubation.

Statistical Analysis. Values of P_{alb} are expressed as mean ± SEM. *N* represents the number of glomeruli studied. Results were compared using ANOVA, and significance was defined as $P < 0.05$.

Results

Effects of Lyophilization of Serum. Biological effects of small molecules can generally withstand lyophilization, storage, and reconstitution whereas many large proteins lose their activities during these treatments. Further, transport and shipment of lyophilized serum specimens is both economical and convenient. Sensitivity of FSPF to lyophilization and reconstitution was studied by determining the change in its effect on P_{alb} before and after lyophilization. P_{alb} of lyophilized specimens averaged 0.69 ± 0.03 ($n = 12$). This value was not different from that of nonlyophilized specimens (0.71 ± 0.03, $n = 12$). Values for paired specimens differed by an average of −0.03 ± 0.02 ($P > 0.3$ by paired *t* test). These results indicate that lyophilization followed by storage at room temperature for 3 days does not alter the glomerular effect of FSPF (Fig. 1).

Chromatographic Characteristics. Interaction of FSPF with several chromatography media was studied for two reasons. First, these studies provide information on some of the important physicochemical properties of FSPF. Second, results may indicate suitable material(s) for removal of FSPF from plasma.

Chromatography with Affinity Media. The FSGS 70% supernatant was passed through affinity media, including (i) Cibacron blue F3GA gel (blue gel), (ii) con A–sepharose gel (con-A), (iii) heparin-agarose, and (iv) agarose- or silica-bound protein A. Bound and unbound fractions were collected and analyzed for FS activity. Buffers used for chromatography are briefly described in Table 1.

FSPF was retained by blue gel and the bound fraction eluted between 0.4 and 1.5 M of sodium chloride in 0.025 M sodium phosphate buffer (Fig. 2a). FSPF was not retained by con-A (Fig. 2b) and was detected in the unbound fraction. FSPF did not bind with heparin-agarose (Fig. 2c) and was largely present in the unbound fraction.

FSPF was retained on agarose-bound protein A (Fig. 2d) and partially eluted with 0.025 M citrate (pH 2.4), whereas washing with 1 M sodium thiocyanate completely eluted FSPF. Identical results were obtained using silica-bound protein A and are not presented. Only about 4% of the total proteins in the 70% ammonium sulfate supernatant were retained by protein A column. Protein A affinity chromatography resulted in a 10,000-fold enrichment of FSPF (Table 2).

Chromatography with Ion Exchange Media. The FSGS 70% supernatant was passed through a column containing cationic (Mono-S) or anionic (DEAE-Sephacel and Mono-Q) chromatography medium. Bound and unbound fractions were collected and tested for the FS activity using the *in vitro* assay. FSPF did not bind with Mono-S and was present in the unbound fraction (Fig. 3a); the bound fraction eluted with 1 M sodium chloride in MOPS buffer (pH 7.4) did not increase P_{alb}. FSPF did bind with DEAE-Sephacel and Mono-Q. The bound factor eluted with 0.1 M

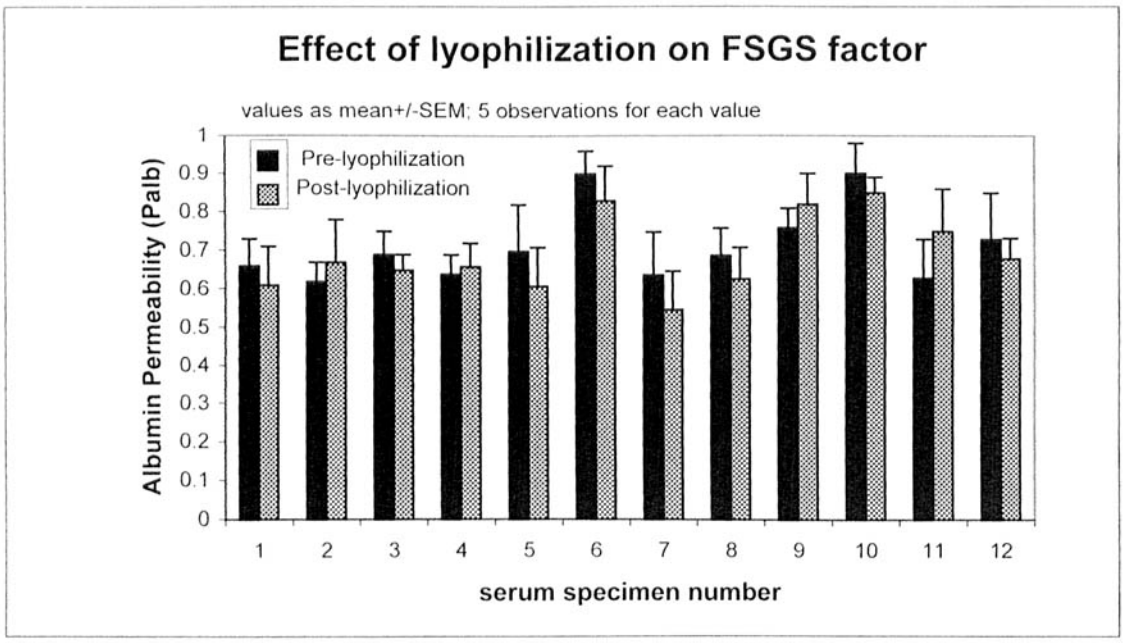

Figure 1. Lyophilization does not alter the glomerular effect of FSPF. We tested the hypothesis that lyophilization does not change the glomerular permeability caused by FSPF. Serum samples from 12 patients with recurrent FSGS were lyophilized, stored at room temperature (21°C) for 3 days, and reconstituted for permeability assay as described in *Materials and Methods*. Freshly thawed aliquot of serum was used as control for each specimen. Average P_{alb} of lyophilized serum was 0.69 ± 0.03, and that of nonlyophilized specimens was 0.71 ± 0.03. Values for paired specimens differed by an average of −0.03 ± 0.02 ($P > 0.3$ by paired *t* test), indicating that lyophilization followed by short-term storage of serum does not alter FSPF with regard to its effect on glomerular albumin permeability.

sodium chloride in 0.01 M Bis-Tris, pH 6.5, from DEAE-Sephacel (Fig. 3b) and with 0.5 M sodium chloride in 0.01 M Bis-Tris, pH 6.5, from Mono-Q column (Fig. 3c). As shown in Table 2, about 80% of the total protein in the 70% ammonium sulfate supernatant was retained by the Mono-Q column and eluted with 0.5 M sodium chloride. Thus, the use of low-pressure Mono-Q anion exchange chromatography did not increase the purity of the protein preparation that contains FSPF. However, it demonstrated an important characteristic of the active agent that interacts with glomerular membrane(s).

***In Vivo* Dose Response.** We attempted to differentiate between the amount of FSPF required to increase P_{alb} and that needed to induce proteinuria. P_{alb} of glomeruli isolated 24 hrs after intravenous injection of FSPF was increased after doses of 3–12 mg of protein. P_{alb} was not increased by injection of NPP 70% supernatant or by 1 mg FSGS 70% supernatant. Increased P_{alb} was noted 3 hrs after intravenous injection of FSPF at 12 mg/rat (FSPF 0.59 ± 0.05, $n = 10$, vs. normal pooled plasma, 0.09 ± 0.11, $n = 15$, $P < 0.05$). Proteinuria was not evident after injection of 1, 3, or 6 mg of FSGS 70% supernatant protein. Proteinuria occurred only after injection of 9 or 12 mg of FSGS 70% supernatant and averaged 10.2 ± 2.7 mg/mg creatinine ($n = 6$) in 24-hr urine samples. Urinary protein in rats treated similarly with a normal plasma fraction averaged 2.8 ± 2.1 mg/mg creatinine ($n = 4$). These findings were similar to previously reported increase where we also found that proteinuria is evident between 6 and 24 hrs after injecting FSPF (33, 34). The discordance of increased P_{alb} and proteinuria suggested that P_{alb} is a more sensitive measure of glomerular injury by FSPF. Additional factors, such as injury that impairs tubular reabsorption, alteration in hemodynamic factors, or other compensatory mechanisms, may be required for the development of chronic proteinuria (Fig. 4).

Time Course of *In Vitro* Change in P_{alb}. In order to determine the minimum time required for increase in P_{alb} by FSPF, isolated glomeruli were incubated with FSPF for 2–10 mins. Earlier time points were not tested because of time restrictions in performing the necessary steps for videomicroscopy. P_{alb} was increased to 0.78 ± 0.09 after 2 mins of incubation with FSPF (10 μg/ml total protein). P_{alb} remained elevated at 3, 4, 6, 8, and 10 mins of incubation, with P_{alb} of 0.80 ± 0.06, 0.70 ± 0.07, 0.67 ± 0.07, 0.74 ± 0.08, and 0.76 ± 0.07, respectively. Thus, the increase that we have measured after 10 mins, our usual duration of incubation for studies of FS activity, represents a change that has reached completion as early as 2 mins (Fig. 5).

***In Vitro* Change in Glomerular Protein Tyrosine Phosphorylation.** In order to determine the relationship between protein phosphorylation/dephosphorylation and increased P_{alb}, we studied the effect of FSPF on protein tyrosine phosphorylation. Western blotting of total glomerular proteins using monoclonal antiphosphotyrosine antibody showed decreased phosphorylation of several proteins after 3 mins of incubation with FSPF that coincided with increased P_{alb} (Fig. 6). Tyrosine phosphorylation of FAK

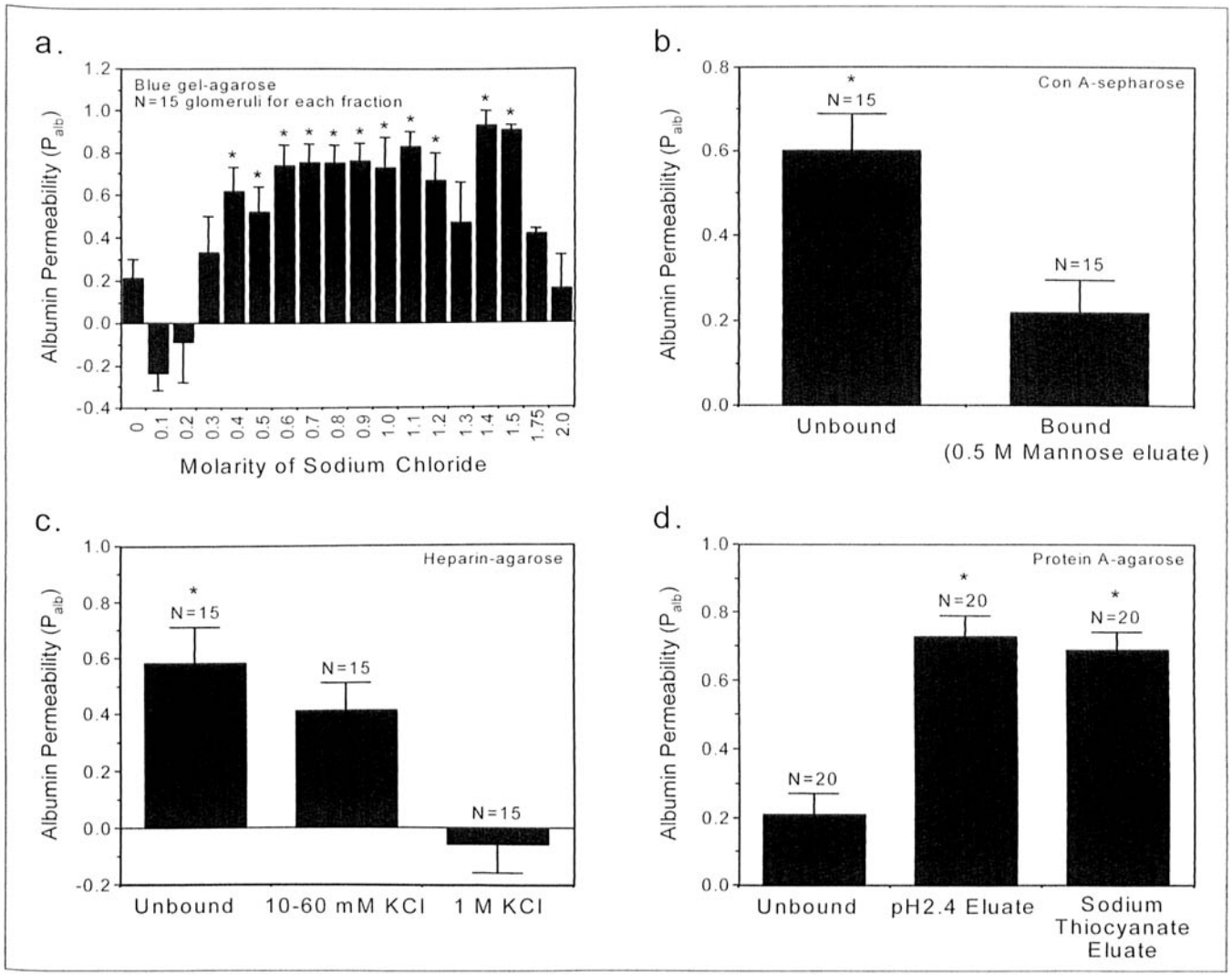

Figure 2. Binding characteristics of FSPF to affinity media. We hypothesized that certain physicochemical characteristics of FSPF may be determined by understanding its binding with chromatography materials. FSPF in the 70% supernatant was passed through (a) Cibacron blue F3GA gel, (b) con A–sepharose gel, (c) heparin-agarose, and (d) agarose- or silica-bound protein A affinity media. Bound and unbound fractions were tested for capacity to increase P_{alb} (FS activity). FSPF was retained by blue gel, did not bind to con A or heparin-agarose, and was retained on either protein A. Values for P_{alb} are expressed as mean ± SEM. $P_{alb} \geq 0.5$ was considered as a significantly different increase in P_{alb} over the control and is indicated by an asterisk. *N* represents the number of glomeruli studied.

and paxillin were decreased after 3 mins of incubation with FSPF. Tyrosine phosphorylation of additional unidentified proteins was also decreased at this time. Representative gels from six analyses are shown in Figure 7. These findings are consistent with the hypothesis that FSPF increases P_{alb} via pathways involving activation of tyrosine phosphatase or inactivation of tyrosine kinases in glomerular cells.

Effect of Inhibitor of Tyrosine Phosphatase on Altered P_{alb}. The rapid increase in P_{alb} may be mediated by one or several signaling pathways in glomerular cells. In these experiments we sought to determine the potential role of protein tyrosine phosphorylation in FSPF-induced increase in glomerular permeability. P_{alb} after incubation with FSPF significantly increased compared to control (FSPF 0.88 ± 0.09, $n = 5$, vs. control, 0.01 ± 0.01, $n = 5$, $P < 0.001$). Tyrosine phosphatase inhibition prevented the increase in P_{alb} ($P_{alb} = 0.26 \pm 0.03$, $P < 0.001$, vs. FSPF). Results are shown in Figure 8. These results indicate that phosphorylation/dephosphorylation may play a role in FSPF-induced increase in glomerular permeability.

Discussion

Focal segmental glomerulosclerosis (FSGS) is clinically diagnosed by characteristic focal and segmental changes in glomerular histology. FSGS develops as a secondary complication in a large number of pathological conditions. The underlying biochemical mechanisms leading to these changes are not clear. Our studies have been aimed at understanding the mechanism of recurrent proteinuria and FSGS after transplantation. Studies from our laboratory and the laboratories of other investigators have confirmed the

Table 2. Purification by Mono-Q Anion Exchange and Protein A Affinity Chromatography Techniques

Fraction	Total protein (mg)	Total protein (% of protein in plasmaheresis fluid)	Minimum protein (μg) required for $P_{alb} \geq 0.5$	Fold purification[a]
Plasmapheresis fluid[b]	100,000	100	100	1
70% ammonium sulfate supernatant[c]	1500	1.5	1	100
Mono-Q anion exchanger[d]	1227	1.22	1	100
Protein A[e]	55	0.055	0.01	10,000

[a] Fold purification = minimum protein required to obtain $P_{alb} \geq 0.5$ in the plasmapheresis fluid divided by the amount of protein required at each stage of purification.
[b] Plasmapheresis fluid means plasmapheresis fluid specimen. Typical starting volume of plasmapheresis fluid was about 4,000 ml. The amount of protein ranged from less than 30 mg/ml to about 45 mg. Data have been normalized to 100,000 mg total initial protein.
[c] 70% ammonium sulfate supernatant means the supernatant at 70% ammonium sulfate saturation.
[d] Mono-Q anion exchanger means the total bound protein fraction eluted with 0.5 M sodium chloride.
[e] Protein A means the total bound fraction from protein A affinity column eluted with sodium citrate buffer (pH 2.4) followed by sodium thiocyanate. Averages of values obtained by using silica-bound protein A and agarose-bound protein A are presented.

presence of a substance in the plasma, FSGS permeability factor (FSPF), which increases glomerular albumin permeability (24, 29–35).

To date, we have identified FSPF as a protein associated with a low-molecular-weight fraction from plasma (33, 34) and have shown that activity in our *in vitro* assay is correlated with the severity of disease and risk of recurrence after transplantation (32, 49). Permeability activity is blocked by components of normal plasma (35, 36), cyclosporine A (50), indomethacin (51), or specific glycosides derived from *Trypterygium wilfordii* (52).

Despite this correlation and our finding that FS permeability activity is greater in patients with severe and rapidly progressive or recurrent disease, we have found instances in which proteinuria was unaffected by removal of activity with plasmapheresis (53). We interpret this persistence of proteinuria as evidence of causes of proteinuria that are independent of activity; these may include glomerulosclerosis with fixed anatomical changes in the glomerular capillaries. In another independent series of patients with FSGS and steroid-resistant proteinuria, full or partial remission occurred during treatment with cyclosporine A despite continuing FS permeability activity (54). We interpret this as support of our prior observations that cyclosporine A has a direct protective effect of the capillary permeability barrier. Our work regarding the FSGS factor has been recently summarized (55).

Others have employed methods derived from our initial protocols for measuring P_{alb} (29, 36, 56–58) and have confirmed the association between P_{alb} and recurrence following transplantation in children (56) and the presence of blocking substances in normal plasma (36) and have described several components of fractions comparable to the FSGS 70% supernatant employed in the current studies (57–60). Additional reports regarding permeability activity in sera from patients with genetic abnormalities in podocin (61) and the presence of a blocking activity in the urine of patients with idiopathic FSGS are of interest (62). However, it is not currently possible to determine whether the substance(s) responsible for these actions correspond to those that we are studying. Recently, increased interest in this area and the complexity of the problem emphasize the need to study each variant of FSGS individually and to the common characteristics among them.

Previously, we demonstrated that serum/plasma or partially purified FSPF in 70% ammonium sulfate supernatant from 15 individuals increased P_{alb} and was susceptible to heat and proteolytic enzyme (33). In the present studies, we observed that lyophilization followed by storage at room temperature and reconstitution 3 days later did not alter the glomerular effect of FSGS serum (Fig. 1). P_{alb} of glomeruli treated with serum before or after lyophilization was not significantly different ($P > 0.3$, $n = 12$). Lyophilization may alter secondary and tertiary structures of many macromolecules and result in altered biological properties (63–67). Preservation of biological activity and antigenicity of many macromolecules following dehydration may require addition of stabilizers (68, 69). Our experiment using sera from 12 individuals indicates that FSPF withstands lyophilization and storage up to 3 days without significant change in biological activity in terms of its effect on P_{alb}. This observation also suggests that FSPF may not have a complex secondary structure and that FSPF may be a low-molecular-weight molecule(s) (33).

Further information on the physicochemical nature of FSPF was obtained using affinity binding materials as well as anion and cation exchangers. Plasma from each patient was treated separately to avoid confusion based on potential differences among patients. We have followed the active factor by testing P_{alb} under standard conditions and have used the criterion of $P_{alb} \geq 0.5$ to determine the presence of active material, as in prior studies.

The binding and elution pattern of FSPF to Cibacron blue F3GA (blue gel) is similar to that for albumin, which also elutes over a wide range of salt concentration. The blue gel is used for affinity-based removal of albumin (39, 40). These observations are consistent with potential association

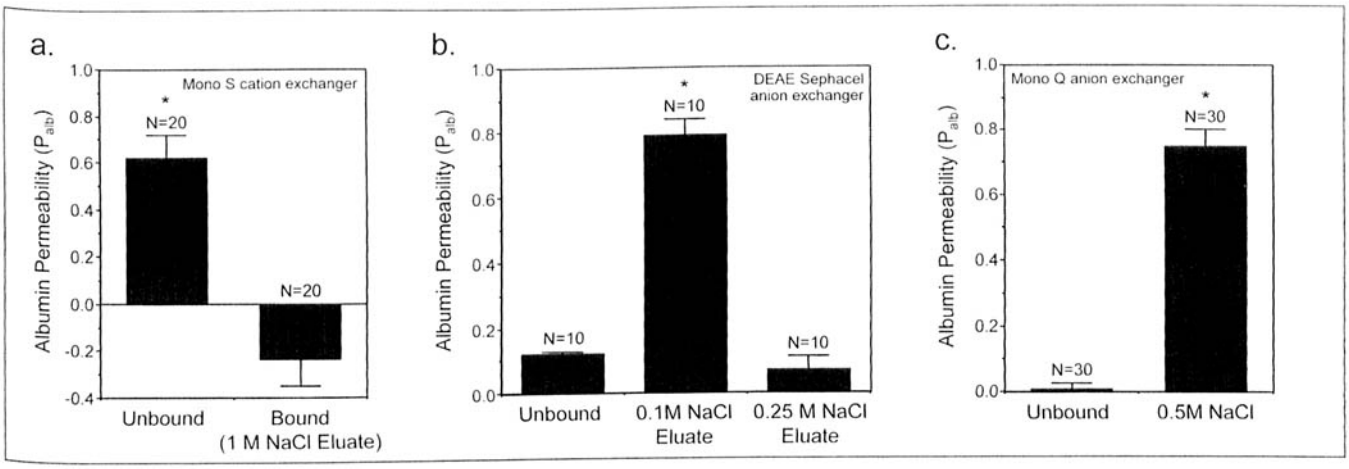

Figure 3. Binding characteristics of FSPF to ion-exchange media. We hypothesized that binding of FSPF to that physicochemical characteristics of FSPF may be determined by using ion-exchange chromatography materials. FSPF in the 70% ammonium sulfate supernatant was passed through (a) cationic (Mono-S) and anionic chromatographic materials, (b) DEAE-Sephacel, and (c) Mono-Q. Bound and unbound fractions were tested for FS activity. FSPF did not bind with Mono-S and was present in the unbound fraction (a), whereas FSPF bound with DEAE-Sephacel and Mono-Q and was eluted with sodium chloride (b and c). Values for P_{alb} are expressed as mean ± SEM. $P_{alb} \geq 0.5$ was considered as a significantly different increase in P_{alb} over the control and is indicated by an asterisk. *N* represents the number of glomeruli studied.

between FSPF and albumin as a carrier molecule. These data demonstrate that we are not able to discriminate between albumin and FSPF and suggest the possibility that FSPF is a small molecule attached to albumin or other larger molecule(s) through hydrophobic interactions. Albumin is known for its role as a carrier molecule (70–72). Although the blue gel may not be useful in purification of the FSGS factor, it has provided helpful clues to the nature of this molecule.

The absence of binding to con A (Fig. 2b) suggests that the factor lacks mannose moieties or that those present are not available to bind with con-A. Results of studies with heparin-agarose matrix (Fig. 2c), cation exchanger Mono-S (Fig. 3a) and anion exchangers, DEAE-Sephacel (Fig. 3b), and Mono-Q (Fig. 3c) are consistent with the presence of anionic rather than cationic charge. Further, anionic nature of FSPF indicates that its potential binding with glomerular cell surface does not depend on charge neutralization. However, Mono-Q appears to retain most of the proteins present in the 70% ammonium sulfate supernatant and shows poor selectivity (Table 2). Elution of bound proteins from Mono-Q matrix by a salt or pH gradient may prove useful in separating the active component from inactive proteins.

Prior studies in our laboratory and those of others indicate that FSPF binds to and can be eluted from protein A (54, 73, 74). Additional findings indicate removal of the permeability factor by immunoadsorption with anti-human immunoglobulins and with low-density lipoprotein apheresis (75, 76). Although protein A immunoadsorption may result in remission in a variety of proteinuric diseases (77), the positive response in patients with FSGS is consistent with speculations that FSPF may have structural similarities to immunoglobulin fragments (33, 78). Several proteins in the fraction retained by and eluted from protein A have been identified, but none of these appears to represent the active molecule (58–60). Our results show that protein A may serve as a useful medium to remove the majority of inactive proteins from the 70% ammonium sulfate preparation (Table 2).

Injection of plasma fractions results in consistent proteinuria in rats and the proteinuric activity is correlated with *in vitro* activity of the preparation used. We have reported a rapid clearance of injected FSPF from plasma (34), neutralization of FS activity by normal plasma (35), transient albuminuria, and proteinuria several hours after injection of FSPS (33, 34). These observations led us to consider the increase in glomerular permeability and the onset of proteinuria as two distinguishable events and to investigate the possibility that FSPF may alter the glomerular permeability characteristics and cause proteinuria in a dose-related manner.

The current report expands on prior studies by defining the relationship between the dose of FSPF administered and the proteinuric response after injection and by documenting the time course of the permeability response *in vitro*. Glomerular P_{alb} was increased in isolated from rat kidneys 24 hrs after injection of FSGS 70% ammonium sulfate supernatant, confirming the results of our prior studies (34). The specificity of this response is shown by the absence of increased P_{alb} after injection of an identically prepared fraction from normal pooled plasma. The P_{alb} was increased in response to as little as 12 mg/kg body weight FSGS 70% supernatant, while a larger dose (36–48 mg/kg) was required to produce proteinuria (Fig. 4). The difference between the dose required to increase P_{alb} and that which causes proteinuria is consistent with the fact that proteinuria represents the net effect of protein filtration and tubular reabsorption. This difference in sensitivity provides a potential explanation for the difficulty others have had in using induction of proteinuria as an assay for a putative FSPF and

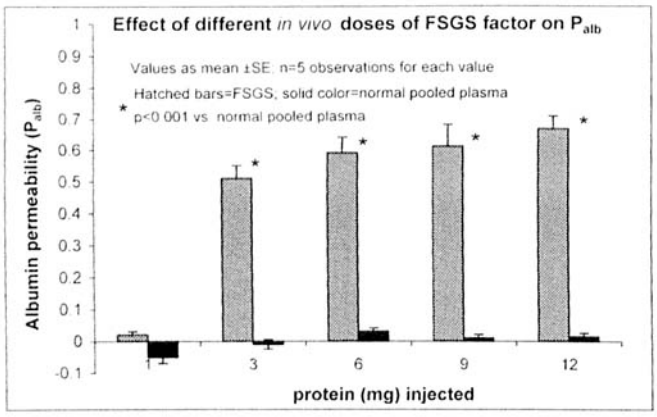

Figure 4. Effect of *in vivo* administration of FSPF on P_{alb} and proteinuria. In order to find out if increased P_{alb} and proteinuria are dose-dependent sequential effects of FSPS, rats were injected 1, 3, 6, 9, or 12 mg protein/rat (approximately 4–48 mg/kg body weight) intravenously. Control rats were treated identically with a preparation from normal pooled plasma. Urine protein and creatinine and glomerular P_{alb} were determined at 24 hrs. Single injection of 1 mg protein did not appear to cause any change in P_{alb} or urinary protein. A dose of 3, 6, 9, or 12 mg protein caused increase in P_{alb}. Urinary protein ($U_{protein}/U_{creatinine}$) was increased only after administration of 9 or 12 mg protein. These findings indicate that an increase in urinary protein may require saturation of tubular capacity to reabsorb increased protein in the glomerular filtrate.

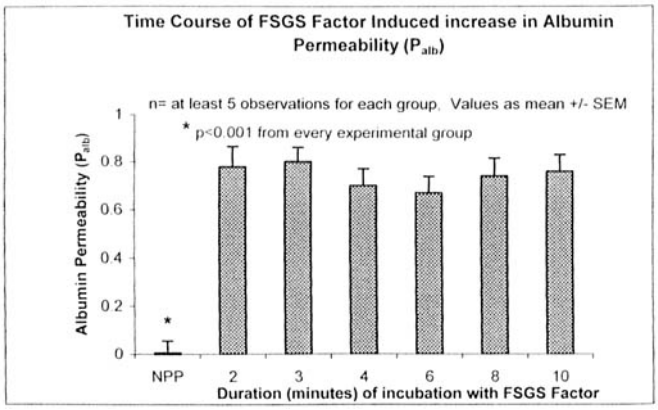

Figure 5. Time course of FSPF induced increase in glomerular albumin permeability (P_{alb}). In order to determine the endpoint at which FSPF-induced increase in P_{alb} can be demonstrated, isolated glomeruli were incubated at 37°C for 2–10 mins with serum from the same patient with recurrent FSPF (20 μg protein/ml) followed by determination of P_{alb} as described in *Materials and Methods.* Glomeruli incubated (10 mins, 37°C) with an identically prepared fraction from normal pooled plasma were used as control. Maximum increase in P_{alb} was attained after 2 min of incubation (0.78 ± 0.09, $P < 0.001$ vs. normal pooled plasma) and was not different from that after 10 mins of incubation.

confirms the potential limitations of injection and measurement of proteinuria to guide purification protocols (74). We postulate that the reabsorptive capacity of the tubule must be exceeded before protein is found in the urine. This may arise from saturation of receptors or endocytotic mechanisms or from tubular injury that impairs these mechanisms. Further studies will be required to distinguish between these possibilities. In related studies, we are attempting to determine if increased P_{alb} and proteinuria are chronologically distinct events. The idea that glomerular injury may precede proteinuria is supported by our preliminary results that demonstrate increased P_{alb} at 3 hrs after injection of FSPF. We have previously shown that serum from FSPF-injected rats does not increase glomerular protein permeability at 3 hrs postinjection and that proteinuria is evident only at 6 hrs postinjection (34). Thus, increased P_{alb} and proteinuria appear to be two distinct but related effects of FSPF that depend on the amount of and the interval after injection of FSPF. The association between increased glomerular permeability, transient proteinuria, and changes in glomerular structure are not defined at the present time. Since the clinical features and histological changes associated with FSGS develop over very extended periods of time, microscopic changes in the rat glomerulus may appear many weeks after repeated administration of FSPF. Therefore, we speculate that increased permeability and transient proteinuria in rats after a single dose of FSPF indicate changes in the functional states of the filtration barrier proteins. Long-term effects of single or multiple doses of FSPF on glomerular histology and the underlying molecular mechanisms have not been studied.

A very rapid permeability response during *in vitro* studies, with maximal increase in P_{alb} after only 2 mins (Fig. 5), points to signal transduction in glomerular cells as the mediator of increased permeability. Signal transduction through pathways including activation of phospholipase A_2, eicosanoid synthesis, phosphorylation of intracellular proteins, signaling by cyclic nucleotides, or changes in ionized calcium have each been described in glomerular epithelial cells (50, 79, 80). Each is a candidate for mediating increased permeability. In the current studies, we have shown significant changes in tyrosine phosphorylation of FAK, paxillin, and a number of other proteins after exposure to FSPF (Figs. 6–8). These changes in phosphorylation are analogous to those shown by others during increased P_{alb} in response to nitric oxide (57). A dynamic equilibrium in the phosphorylated and dephosphorylated forms of cellular proteins appears to be critical for a large number of biological phenomena, including the control of tight junctions in epithelial cells (81–84) and focal adhesion and barrier function of endothelial cells (85–87). Cellular response to environmental changes is initiated by biochemical signals between and within cells. Cell-matrix interactions affect gene expression, ion movement, and activation of signaling pathways and are indicated by early changes in tyrosine phosphorylation of proteins. Phosphorylation of FAK, a nonreceptor kinase, represents an early event in integrin-dependent organization of adhesion structures (88). Phosphorylated FAK interacts with Src kinases that contain SH_2 domains, phosphatidylinositol (PI3) kinase, phospholipase 3 (PLCγ) adapter protein Grb7, FAK phosphorylates paxillin, and $p130^{cas}$, which in turn recruit additional signaling molecules resulting in activation of downstream pathways (89–92).

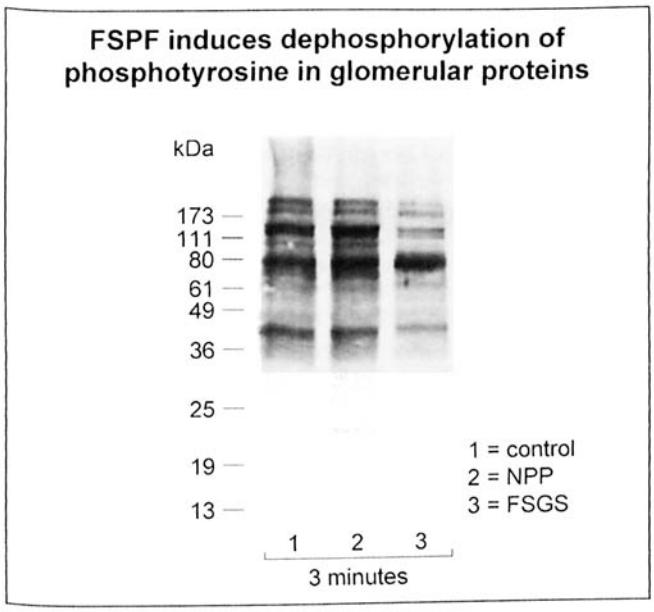

Figure 6. Western blotting of phosphotyrosine in total glomerular protein. We hypothesized that FSPF-induced P_{alb} rapid increase may be due to changes in pathways that are regulated by phosphorylation/dephosphorylation of tyrosine residues in certain proteins. Isolated glomeruli were incubated with (1) normal saline, (2) purified fraction from NPP (20 μg/ml) prepared identically as FSPF, or (3) FSPF (20 μg/ml) prepared from FSGS plasma for 3 mins at 37°C using buffers and conditions as described in *Materials and Methods*. Glomeruli were treated with a lysis buffer containing orthovanadate and homogenized as described in *Materials and Methods*. Total protein content was determined by spectrophotometry. Proteins were separated by SDS-PAGE using a 4%–20% Tris-Glycine gel followed by electrotransfer to a nitrocellulose membrane. Membranes were blocked with 5% fat-free milk and probed with an antiphosphotyrosine monoclonal antibody followed by detection using HRP-conjugated rabbit anti-mouse IgG and ECL reaction. A typical gel is presented. FSPF induces dephosphorylation of phosphotyrosine in a large number of glomerular proteins within a time period that coincides with increased P_{alb}.

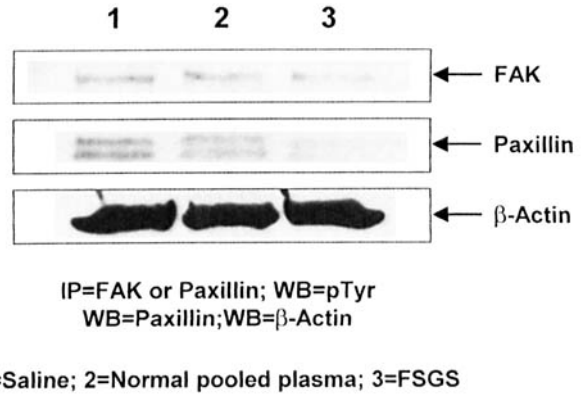

Figure 7. Effect of FSPF on tyrosine phosphorylation of FAK and paxillin. Rapid dephosphorylation of tyrosine occurred in FAK and paxillin, a protein that is phosphorylated by FAK and is linked with several kinases and cytoskeletal proteins. Isolated glomeruli were incubated with (1) normal saline, (2) purified fraction from NPP (20 μg/ml) prepared identically as FSPF, or (3) FSPF (20 μg/ml) prepared from FSGS plasma for 3 mins at 37°C using buffers and conditions as described in *Materials and Methods*. Glomeruli were treated with a lysis buffer containing orthovanadate and homogenized as described in *Materials and Methods*. Total protein content was determined by spectrophotometry. Immunoprecipitation using anti-FAK or anti-paxillin antibody, SDS-PAGE using 4%–20% gradient (FAK) or 12% Tris-glycine minigel, electrotransfer to nitrocellulose membranes, immunoblotting using mouse antiphosphotyrosine antibody followed by HRP-conjugated rabbit anti-mouse IgG antibody, and ECL-based detection followed by image recording on a photography film were carried out as described in *Materials and Methods*. A typical gel is presented. Decreased tyrosine phosphorylation of both FAK and paxillin was evident, indicating that FSPF-induced increase in glomerular permeability involves changes in cell-matrix adhesion and changes in cytoskeletal organization.

We postulate that diminished cell-cell interactions via the slit-pore junction or impaired cell-matrix attachment are integral to increased glomerular protein permeability. Since we have previously shown that alteration of eicosanoid synthesis by cyclooxygenase inhibition prevents FSPF-induced increase in P_{alb} (51), we propose that more than one signaling pathway may be involved in increased permeability.

In summary, the current studies provide new information concerning the nature of FSPF and potential mechanisms of its effect on glomerular permeability. FSPF appears to be bound to albumin and other plasma proteins and carries an anionic rather than a cationic charge. The resistance of FS activity to lyophilization suggests that it is a small molecule. Binding of FSPF with protein A is consistent with the postulate that it may share structural features with immunoglobulins. We have confirmed the sensitivity of the *in vitro* P_{alb} assay and its utility in developing protocols for purification. Insights regarding potential cellular mechanisms of action arise from observations that the increase in P_{alb} during *in vitro* studies is maximal within 2 mins, and is associated with alterations in tyrosine phosphorylation of cellular proteins, including FAK and paxillin, and is prevented by inhibition of tyrosine phosphatases. These findings strongly implicate intracellular signaling pathways as mediating the effect of FSPF, particularly pathways that involve phosphorylation cascade(s). These data and the binding with anion exchangers indicate that FSPF does not cause glomerular changes by charge neutralization. Our results point the way for future studies of the interaction between the permeability responses and cell detachment with podocyte loss, altered matrix synthesis and turnover, and apoptosis. The fact that increased P_{alb} after systemic infusion occurs at doses lower than those required for proteinuria confirms the sensitivity of the assay for P_{alb} and the potential advantage of its measurement to guide strategies for purification of FSPF.

Taken together, the current observations support the theses that development of chronic proteinuria is a complex process that involves the entire nephron and that changes in albumin permeability represent a glomerular event that can be observed by lower doses of FSPF compared to those required to cause proteinuria and involves changes in the

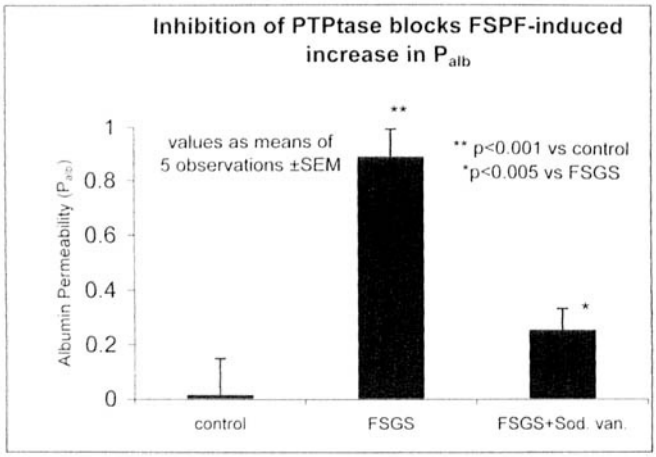

Figure 8. Phosphotyrosine phosphatase (PTPtase) inhibitor protects glomeuruli from FSPF-induced increase in P_{alb}. We tested the hypothesis that dephosphorylaton of protein tyrosine is involved in FSPF-induced increase in P_{alb}. Isolated glomeruli were incubated with FSPF (20 μg/ml) in the presence of sodium orthovanadate (200 μM) for 10 mins followed by determination of P_{alb}. Glomeruli treated with FSPF alone were used as positive control. Inhibition of PTPtase provided significant but incomplete protection against FSPF-induced increase in P_{alb}. FSPF with sodium orthovanadate, 0.25 ± 0.03, versus FSPF alone, 0.88 ± 0.09, $P < 0.005$). These findings indicate that signaling pathways that involve protein phosphorylation/dephosphorylation are involved in FSPS-induced increase in P_{alb}.

cytoskeleton and cell-matrix or cell-cell interactions. The combined use of biochemical techniques for protein purification and measurement of P_{alb} is useful in defining the characteristics and potential mechanisms of action of FSPF. The 70% ammonium sulfate fraction and its derivatives will provide material for additional proteomic analyses. Understanding the affinity properties of FSPF will provide the basis for design of interventions to remove it from the circulation of patients at risk for recurrent FSGS. Identification of cell signaling pathways that affect increased permeability opens the way for studies of mechanisms of action and for therapy to prevent or reverse proteinuria.

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