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Apolipoprotein E in idiopathic nephrotic syndrome and focal segmental glomerulosclerosis

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Background. Hyperlipemia characterizes nephrotic syndrome (NS) and contributes to the progression of the underlying nephropathy. The data in the literature support an implication of apolipoprotein E (apoE) in both hyperlipemia and focal segmental glomerulosclerosis (FSGS), a malignant condition associated with NS.

Methods. The apoE genotype was determined in 209 nephrotic patients, who were classified according to age and their response to steroids as resistant children ($N = 96$) and adults (43), and steroid dependent (33) and steroid responder (37) children. A total of 123 presented the histological features of FSGS. In a subgroup of 28 patients, serum and urinary levels of apoE and renal deposits were evaluated by immunofluorescence.

Results. The allelic frequencies of the three major haplotypes ε_2, ε_3, and ε_4 were the same in nephrotic patients versus controls, and homozygosity for $\varepsilon_3\varepsilon_3$ was comparably the most frequent genotype (70 vs. 71%) followed by $\varepsilon_3\varepsilon_4$, $\varepsilon_2\varepsilon_3$, $\varepsilon_2\varepsilon_4$, $\varepsilon_4\varepsilon_4$. Serum levels of apoE were fivefold higher in NS and in FSGS patients than in controls, with a direct correlation with hypercholesterolemia and proteinuria. ApoE genotypes did not influence serum levels. Urinary levels were 1/10,000 of serum with an increment in nephrotic urines. Finally, immunofluorescence demonstrated the absence of apoE in sclerotic glomeruli, while comparably nephrotic patients with membranous nephropathy had an increased glomerular expression of apoE.

Conclusions. ApoE is dysregulated in NS with a marked increment in serum, which is a part of the complex lipid metabolism. Down-regulation of glomerular apoE instead is a peculiarity of FSGS and may contribute to the pathogenesis of the disease. The normal distribution of apoE genotypes in nephrotic patients with FSGS excludes a pathogenetic role of genetic variants.

Severe hyperlipemia is a constant feature of nephrotic syndrome, which is characterized by high cholesterol, triglyceride, and low-density lipoprotein (LDL) levels, while the high-density fraction appears stable or only slightly decreased [1–3]. The predominant idea is that high cholesterol and LDL levels negatively influence the outcome in these patients [4], but the mechanisms remain hypothetical.

Apolipoprotein E (apoE) is an amphipathic protein that plays an essential role in maintaining lipid homeostasis brought about by the regulation of lipoprotein trafficking as well as cholesterol absorption and excretion [5, 6]. A second major function of apoE is renal protection, since it regulates mesangial cell proliferation and matrix expansion [7]. It is not surprising that mice lacking apoE for genetic manipulation develop atherosclerosis and renal lesions [8, 9] resembling glomerulosclerosis (FSGS). Therefore, low apoE may affect the plasma lipid profile, while low glomerular apoE expression may affect mesangial cell proliferation and eventually be a determinant of the accumulation of matrix.

Not all apoE isoforms possess these functions, however, since the rapid clearance of lipoproteins depends on the presence of the Arg 158 residue, while a Cys in position 112 mediates binding to high-density lipoprotein (HDL). $ApoE_3$ is the unique subtype that carries both amino acid residues and, therefore, the presence of homozygosity for the $apoE_3$ genotype is considered as the most protective against an altered lipid profile [10]. Studies of a limited number of patients with FSGS indicate

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an association of the two less frequent alleles ε_2 and ε_4 with FSGS [11, 12] or, more generally, with nephropathies evolving to renal failure [13]. Moreover, the presence of molecular variants of apoE, such as Sendai and/or others, is associated with modifications of lipid composition and glomerulosclerosis [14–18].

Despite the results of these background studies supporting a role of apoE levels and/or variants in FSGS, no data on apoE levels and renal expression are available. Moreover, only very limited data on apoE genotypes in glomerular disease and specifically in FSGS have been published. Both the quantity and the genotype of apoE could play important roles in the pathogenesis of FSGS, since the inhibitory effect on mesangial proliferation and matrix production could be linked to the amount of apoE, while the presence of genetic variants may confer genetic predisposition to progressive glomerulopathies. The lack of data in this field is probably related to the difficulty in routinely determining apoE levels in serum and, for this reason, we developed a new technique for determining apoE based on dot-blot and immunostaining with specific anti-apoE monoclonal antibodies. The present study is aimed at evaluating the following points: (*1*) apoE genotype in a statistically significant population of children and adults with FSGS enrolled in Italy; (*2*) apoE levels in a cross-section of nephrotic patients, some with histological evidence of FSGS, and association with genotypes; (*3*) the relationship between apoE levels and serum lipids and, finally, (*4*) the glomerular expression of apoE with immunofluorescence. In the first approach we enrolled a sufficient number of patients to allow comparison between children and adults with corticoresistant NS, since they are actually considered different pathological entities. For the quantitative approach, a cross-section of the above patients with well-defined clinical characteristics was utilized, including patients with FSGS, and they were compared with other proteinuric pathologies such as membranous nephropathy (MN). Finally, studies on renal expression of apoE were performed in FSGS patients and for comparison in MN.

METHODS

Patients for apoE genotyping

The patient population for the study of apoE genotypes consisted of 209 children and adults with nephrotic syndrome (Table 1), who had presented moderate to severe proteinuria and had been treated with standard therapeutic protocols according to their sensitivity to different drugs. The standard approach consisted of prednisone (2 mg/kg for 30 to 60 days). In non-responders (partial or global) steroids were associated or substituted with cylophosphamide (2 mg/kg for 60 days) or with cyclosporine (5 mg/kg starting dose, followed by tapering). Patients with steroid-cyclosporine resistance were treated with pulse methylprednisolone (10 mg/kg, 6 cycles). The relevant clinical (gender, age, age at onset of proteinuria, evolution toward renal failure) and pathological features are reported in Table 1, which shows a classification of the different groups according to steroid sensitivity: 139 patients presented strict steroid resistance, 33 were steroid-dependent and 37 responded to one or more steroid regimens. Forty-three of the corticoresistance cohort were older than 18 years at onset of proteinuria, and were considered as a separate group in the statistical analysis. Histology was available for 163 patients. Overall, 123 patients had a diagnosis of FSGS based on the presence of at least one segmental area of glomerulosclerosis in their biopsy, 25 presented mesangial IgM deposition and 14 had no lesions. Most FSGS patients presented persistent resistance to corticosteroids following the reported scheme, but cases of steroid-sensitive FSGS also were observed in the variant of frequent relapses of proteinuria. The apoE genotype was evaluated also in 127 normal controls enrolled in our region plus 398 Italian subjects reported by another group [19].

Table 1. Clinical and pathological features in 209 children with nephrotic syndrome who were enrolled for studying the apoE genotype

	Steroid resistants (N = 139)	Steroid dependents (N = 33)	Steroid responders (N = 37)
Sex	M 82/F 57	M 17/F 16	M 26/F 11
Age at proteinuria			
>18 years	43	—	—
<18 years	96	33	37
Sensitivity to Cys	19/58	17/19	10/10−
Histology available	131	15	17
FSGS	106	6	11
Mes IgM	15	7	3
MCN	10	1	3

Patients were subdivided according to the response to steroids as resistant, dependent or responder. Several patients of the three groups also were treated with cyclosporine with variable response. Most of the steroid resistant patients presented the histology of FSGS (106), Mesangial IgM (15) and minimal change nephropathy (10), but FSGS also was observed in the other groups. Abbreviations are: CsA, cyclosporine; FSGS, focal segmental glomerulosclerosis; Mes IgM, mesangial proloiferating glomerulonephrites with IgM deposition; MCN, minimal change nephropathy.

Patients for the apoE biochemical and immunopathologic study

Serum levels of apoE were determined in 60 patients of the group described in the last section at different stages of the disease and irrespective of the disease activity, which meant that for a few of them more than one serum sample could be checked. These patients were utilized to define any correlation between serum levels and genotypes and/or with proteinuria. In a cross-section of the patients, serum and urine were obtained at disease onset or at recurrence, which then were utilized for biochemical analysis (Table 2). They comprised 28 children,

Table 2. Clinical features and laboratory data of 20 children with idiopathic nephrotic syndrome and 8 with FSGS

Patient number	Sex	Age (years)	Age at onset (years)	Proteinuria g/day	Serum ApoE μg/mL	Creatinine mg/dL	Serum cholesterol mg/dL	Serum triglycerides mg/dL	Therapies
Idiopathic nephrotic syndrome									
1	M	8	6.5	15.0	593.09	0.4	218	119	Pred, statins
2	M	7	5.5	2.0	74.83	0.3	263	98	Pred, CsA, statins
3	M	6	4.5	3.9	238.88	0.3	327	330	Pred, statins
4	M	8	7	2.5	130.18	0.3	114	236	Pred, statins
5	M	10	8	1.0	68.03	0.9	170	170	Nothing
6	F	4	3	30.6	408.06	0.4	114	46	Pred, statins
7	F	3	1.5	10.0	903.62	0.5	492	369	Pred, CsA, statins
8	M	4	3	0.9	116.70	0.4	449	220	Pred, statins
9	M	9	7	6.9	109.41	0.4	314	176	Pred, statins
10	M	7	2	1.0	342.26	0.5	269	106	Pred, statins
11	M	9	4.5	1.7	116.70	0.5	229	162	Pred, statins
12	M	5	3	3.0	210.36	0.3	309	60	Pred, statins
13	F	8	7	10.0	486.58	0.7	491	46	Pred, statins
14	M	4	3.5	1.3	103.98	0.4	273	68	Pred, statins
15	M	5	4	7.8	85.45	0.4	456	374	CsA, statins
16	M	9	8	10.0	223.51	0.7	369	412	Pred, CsA, statins
17	F	5	3.5	2.0	38.30	0.3	180	110	Pred, statins
18	F	7	6.5	5.0	497.25	0.4	425	209	Pred, statins
19	M	6	5	4.0	148.00	1.1	142	108	Pred, statins
20	F	14	12	4.0	342.87		466	121	Pred, statins
FSGS with protenuria									
21	F	6	4	13.0	137.42	0.7	459	540	Pred, CsA, ACE I
22	M	16	8	1.0	626.73	0.9	137	59	Pred
23	M	16	8	2.4	73.07	0.9	170	77	Pred
24	F	11	4	8.2	141.81	7.0	119	517	ACE I
25	M	6	3	14.0	78.21	0.7	325	305	Pred, ACE I
26	M	15	2	3.8	223.85	1.0	236	114	Pred, CsA
27	M	17	8	3.8	113.85	1.1	178	116	Pred, CsA, ACE I
28	M	16	7	1.2	45.91	2.6	192	176	ACE I

Children with idiopathic NS were presenting proteinuria at the time of the enrollment, but after they underwent remission after a regimen with steroids and CsA (**Methods** section). Patients with FSGS presented strict resistance to both drugs and underwent a renal biopsy for diagnostic purpose. Abbreviations are: Pred, prednisone; CsA, cyclosporine; ACE I, angiotensin-converting enzyme inhibitor.

20 with idiopathic NS, that is, acute proteinuria and sensitivity to steroids or to cyclosporine in most cases, and 8 with FSGS; for clinical reasons, the diagnostic approach in patients the former group did not include a renal biopsy. Eight patients presented strict resistance to steroids and cyclosporine at the doses described earlier, and for this reason they underwent a renal biopsy that in all cases demonstrated lesions typical of FSGS (at least one area of segmental or global glomerulosclerosis). The gap between actual age and age at onset was different between groups, because the time lag between onset of proteinuria and follow-up was brief in the former group. All the patients enrolled in this study group were treated or had been treated in the previous six months with steroids associated in some cases with cyclosporine and angiotensin converting enzyme (ACE) inhibitors. Most were treated with 3-hydroxy-3-mathylglutaryl coenzyme A (HMG CoA) reductase inhibitors. Finally, 22 patients with NS and renal histological lesions of membranous nephropathy (aged between 18 and 60 years) were enrolled for evaluating the importance of NS in the regulation of serum apoE. Serum and urine were obtained also from 50 age-paired normal controls to define the reference range of apoE levels in Italy.

Serum, urine and DNA

Sera for apoE measurement were obtained in the morning after an overnight fast, and in parallel a blood sample was collected in ethylenediaminetetraacetic acid (EDTA) for DNA purification. Serum was centrifuged at 1500 × *g* for 15 minutes and then stored at −80°C. On the same day, the first morning urine was collected in sterile vials, and after centrifugation at 8000 × *g* for 10 minutes the supernatants were stored at −20°C. DNA was extracted by affinity column (Boehringer Mannheim GmbH, Mannheim, Germany).

ApoE genotyping

Apolipoprotein E genotypes were determined by multiplex amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) as described by Donohoe et al [20], modified by the use of Perkin Elmer Taq-Gold (1 U with related buffer) and different magnesium chloride and primer concentrations. Accordingly, for detecting Arg 112 and Arg 158, we used 1.8 mmol/L of $MgCl_2$; for Arg 112 and Arg 158, the allele specific-primer concentration was 0.6 μmol/L. For detecting Cys 112 and Cys 158, 1.5 μmol/L of $MgCl_2$ was used, and

the concentration of the allele-specific primer was 0.4 μmol/L for Cys 112, and 0.8 μmol/L for Cys 158. In all reactions the same 0.8 μmol/L common primer and 32 nmol/L α-1-antitrypsin primer concentrations were utilized. Amplification was performed with a Genenco Thermocycler (Model PTC 200).

Antibodies

Monoclonal anti-apoE antibodies (clone 2E1) were obtained from Roche (Mannheim, Germany). Their specificity for apoE was evaluated by immunoWestern blot after two-dimensional electrophoresis of whole serum (Fig. 1), the methodology of which has previously been described in detail [21]. Briefly, the IPG strips were rehydrated overnight at 4°C in 9 mol/L urea, 2% wt/vol CHAPS; 0.6% wt/vol carrier ampholytes with a wide range mixture (60% pH 4 to 8, 40% pH 3.5 to 10) and a trace of bromophenol blue. Proteins, 30 μg, were solubilized with a solution containing 9 mol/L urea, 4% wt/vol CHAPS and 40 mmol/L Tris. Isoelectric focusing was performed at 18°C. The pH gradient was created with Immobilines (IPGs; Amersham Pharmacia Biotechnical, Amsterdam, The Netherlands) pH between 3 and 10 in the first dimension; sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension was performed following the original technique described by Bjellqvist et al [22]. The applied voltage for electrophoresis was increased from 300 to 3500 V during the first five hours, followed by 5000 V for a total of 100 kV h. Before the two dimensional (2-D) run, IPG strips were equilibrated within the strip tray for 30 minutes with a solution of 0.05 mol/L Tris-HCl buffer pH 6.8, 6 mol/L urea, 30% vol/vol glycerol, 2% wt/vol SDS and a trace of bromophenol blue. The second dimension was performed on 180 × 160 × 1.5 mm slabs of polyacrylamide gradient gels (%T 8-16) using piperazine diacrylamide (PDA) as cross-linking agent. The gels were run at 45 mA/gel constant current and maintained at a temperature of 12°C.

For the Western blot, proteins were transblotted to Hybond nitrocellulose membranes (Amersham Pharmacia Biotech) with a Novablot semidry system using a continuous buffer system with 38 mmol/L Tris, 39 mmol/L glycine, 0.035% SDS and 20% methanol. The transfer was achieved at 1.55 mA/cm^2 for 3.5 hours.

Preparation of antibody-enzyme conjugate

Two hundred micrograms of the monoclonal apoE antibodies were coupled to horseradish-peroxidase (HPR) (EC 1829696) according to specific information given by the peroxidase labeling kit (Roche, Mannheim, Germany). The conjugate was mixed with an equal volume of glycerol for storage at −20°C.

Determination of apoE in serum and urine

All samples and the calibrator serum were diluted with TBS pH 7.4 (1:10 vol/vol). A calibration curve was prepared by dilution of purified apoE in TBS at the programmed levels of 25, 50, 100, 200, 400 and 800 μg/mL. Sera and urines were diluted in the same buffer to achieve the desired range of levels, within the standard curves.

For apoE determination we utilized a Bio-Dot apparatus (Bio-Rad, Hercules, CA, USA) following the instruction manual with minor modifications. Accordingly, the nitrocellulose membrane was pre-wetted in TBS and placed on a sheet of Whatman 3MM filter paper soaked in the same solution. After removal by gentle pressure of the air bubbles trapped between the two sheets, the sample template was placed on the nitrocellulose membrane and a vacuum was applied for a few minutes, to fill up the 96 sample wells with 50 μL using a multichannel pipette. The vacuum was applied until all of the samples were adsorbed. The same operation was repeated five times with 150 μL of buffer each, to wash out the non-adsorbed sample. The nitrocellulose then was removed gently.

Hybridization was preceded by incubation with a blocking solution of 0.5% polyvinyl-pyrrolidone mixture (10,000 and 40,000 molecular weight) in 0.1 mol/L acetic acid for one hour at 37°C, which then was removed by washing in 20% Tween-TBS. Incubation with the horseradish peroxidase (HPR)-anti-apoE antibodies complex 0.5 μg/mL in 1% bovine serum albumin (BSA) in TBS was done for two hours at room temperature.

The membrane was then washed with TBT-T four times, 15 minutes each, prior to developing the immunoblot with the enhanced chemifluorescence reagent ECL-Plus kit (Amersham Pharmacia Biotech). The detection of fluorescent signals was acquired with the Optical Scanner Storm 860 with an excitation of λ 420 nm and emission of λ 460 nm.

Validation of the assay was done by determining apoE levels in a standard and in three normal sera at different dilutions (Fig. 2). Coefficients of variation were calculated by repeating apoE determinations at five different dilutions in ten sera.

Immunofluorescence

Apolipoprotein E expression in the kidney was studied by indirect immunofluorescence in renal biopsies of 12 patients with FSGS, 6 with MN and, for comparison, in 12 cadaver kidneys that could not be grafted because of vascular abnormalities.

Tissue samples for light microscopy were fixed in 4% buffered paraformaldehyde and embedded in paraffin. Routine stainings were performed according to standard techniques. For immunofluorescence, the unfixed renal tissue was embedded in OCT compound (Miles Scien-

Table 3. ApoE genotype and allele frequency in a group of 209 nephrotic children who were subdivided according to the clinical response to steroids

	Nephrotic syndrome *N* (%)				
Normals (*N* = 525)	Overall (*N* = 209)	Steroid-sensitive (*N* = 37)	Steroid-dependent (*N* = 33)	Steroid-resistant <18 years (*N* = 92)	Steroid-resistant >18 years (*N* = 43)
ε2ε2 5 (1)	0	0	0	0	0
ε2ε3 44 (8)	23 (11)	2 (5.4)	4 (12.1)	13 (14.1)	4 (9.3)
ε2ε4 10 (2)	6 (2.9)	0	1 (3.1)	3 (3.1)	1 (2.3)
ε3ε3 372 (71)	146 (70)	28 (75.7)	23 (69.7)	67 (72.7)	28 (65.1)
ε3ε4 9 (17)	33 (15.6)	6 (16.2)	4 (12.1)	13 (14.1)	10 (23.3)
ε4ε4 4 (1)	1 (0.5)	1 (2.7)	0	0	0
Allelic frequency					
E2 6%	6.9%	2.7%	9.1%	8.3%	5.8%
E3 84%	83.3%	86.5%	81.8%	83.3%	81.4%
E4 10%	9.8%	10.8%	9.1%	8.3%	12.8%

Our controls consisted of 127 normal people enrolled in our region, plus 398 normal controls from Italy reported in another work [19].

tific, Naperville, IL, USA), snap-frozen in a mixture of isopentane and dry-ice and stored at −80°C. Subsequently, 5 μm sections were placed on slides and stored at −20°C until being immunostained.

Briefly, cryosections were fixed in cold acetone, rinsed, and sequentially incubated with the primary rabbit polyclonal antibody against apoE, followed by fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit secondary antibody (Zymed, Histoline, Milan, Italy). After washing, sections were then mounted with an anti-fading mounting medium (Vectashield; Vector, DBA Italia SRL, Milan, Italy). Specificity of labeling was demonstrated by the lack of staining after substituting phosphate-buffered saline (PBS) and proper control immunoglobulins (Zymed) for the primary antibody.

Statistical methods

Serum concentrations of apoE in patients with idiopathic nephrotic syndrome and FSGS versus controls were compared utilizing one-way analysis of variance (ANOVA). The frequency of different apoE haplotypes in controls and in our cohorts with nephrotic syndrome was compared with chi-square analysis. Data are expressed as the mean ± standard deviation.

RESULTS

ApoE genotypes

Apolipoprotein E genotypes were determined in 209 nephrotic children (*N* = 159) and adults (43) with a net prevalence of FSGS (Table 1) and 127 normal controls. Data on the apoE genotype of 398 normal Italian subjects reported in another study [19] were utilized for statistical purposes. These patients had been subdivided according to the responsiveness to steroids in corticosensitive (*N* = 37), corticodependent (33) and corticoresistant (139). The high number of patients with corticoresistance and FSGS did not represent the effective incidence of the disease in the proteinuric population, but it was determined by the necessity to look for an association between apoE genotypes and glomerulosclerosis, more than between apoE and proteinuria. The group of patients with corticoresistance actually consisted of 96 children and 43 adults (Table 1) who were considered separately for statistical analysis, since corticoresistance and/or FSGS occurring in children and adults is not considered unequivocally the same disease. Renal histology was available in 131 cases, demonstrating FSGS in 106, mesangial proliferation with IgM deposits in 15, and minimal lesions in 10. This is in agreement with the concept that renal histology is variable in these subjects depending on sampling problems, and does not correspond to clinical outcome. The frequency of the three most common apoE alleles—ε_2, ε_3 and ε_4—together with the percentage of homozygous and heterozygous carriers of the different haplotypes are reported in Table 3, which shows the frequencies for every separate group and for age. Overall, the allelic frequencies for the apo ε_2, ε_3 and ε_4 haplotypes in nephrotic patients were 6.9%, 83.3% and 9.8%, respectively, which were very similar to the frequencies in a normal population of 525 Italian subjects. Homozygous carriers of apo ε_3 were by far the most frequent in all groups with nephrotic syndrome (69.9%), followed by heterozygous $\varepsilon_3\varepsilon_4$ (15.8%), $\varepsilon_2\varepsilon_3$ (11%), $\varepsilon_2\varepsilon_4$ (2.9%) and by homozygous $\varepsilon_4\varepsilon_4$ (0.5%) and $\varepsilon_2\varepsilon_2$ (0%). All these frequencies were equally distributed among the subgroups and were not different from controls.

ApoE in serum and urine

Before starting the screening study on apoE levels in serum and urine, we tested the specificity of antibodies (Fig. 1) and validated the new assay for apoE determination based on dot-blot in a subgroup of patients. The results showed a linearity of the assay in a range of apoE levels between 0.1 and 50 μg/mL (Fig. 2); the coefficient of variation was less than 2.5%.

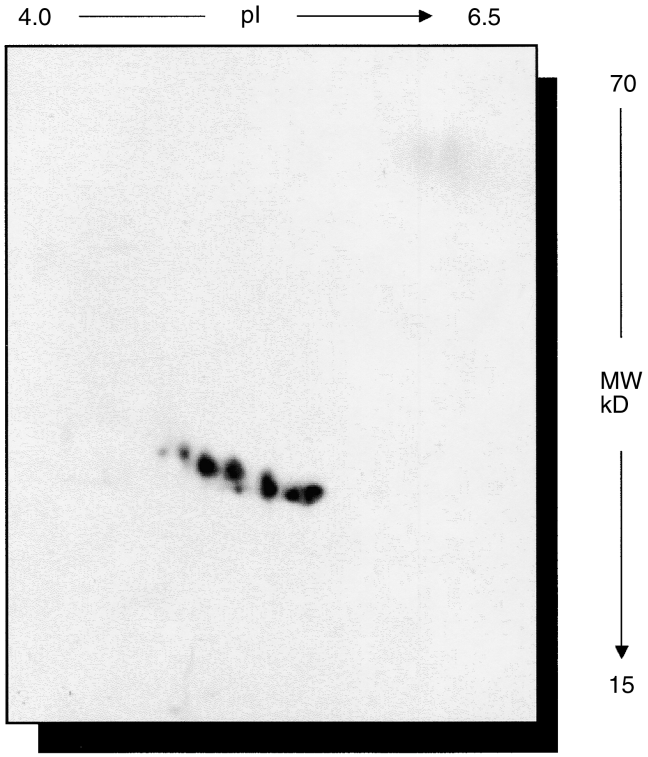

Fig. 1. Specificity of anti-apolipoprotein E (anti-apoE) monoclonal antibodies was evaluated by two dimensional electrophoresis and immunoblot of a normal serum. These antibodies recognized different glycosylated isoforms of apoE. Abbreviations are: pI, isoelectric points; MW, molecular weight.

Serum levels of apoE were first evaluated in a cross-section of 60 patients of the entire cohort to identify any possible correlation between apoE genotypes and levels. Several serum samples were obtained from these patients, at different stages unrelated to disease activity, and were tested to document variability within the same genotype group. There was a random distribution of apoE levels in the groups presenting different genotypes (Fig. 3). It is relevant that considering carriers of the $\varepsilon_3\varepsilon_3$, which is by far the most frequent haplotype, serum apoE varied from the normal range of 40 mg/L up to very high levels of 600 to 800 mg/L. This excludes an influence of genetic factors on serum apoE levels. At the same time, a direct correlation between apoE levels and proteinuria was observed in these patients ($P < 0.001$; Fig. 4).

For 28 children, 20 with idiopathic nephrotic syndrome and 8 with FSGS, serum and urine were obtained at disease onset or during a relapse; frozen sections of the kidney also were available in this group. The clinical characteristics of the 28 patients of this pilot study together with the results on apoE levels are reported in Tables 2 and 4. All but two patients of the FSGS group had normal renal function and all had proteinuria in the nephrotic range. All but three were receiving steroids at the time of the enrollment, in some cases associated with cyclosporine; most patients also were receiving statins. Serum levels of apoE varied between 40 μg/mL (that is, upper limit in normal range) to almost 1000 μg/mL. However, due to the skewed incidence of elevated serum apoE levels among the patients and to the minimal range of variation in the normal population, the difference between nephrotic patients and controls was highly significant (44.6 ± 2.9 μg/mL controls vs. 261.9 ± 222.5 nephrotic and 180.14 ± 188.7 FSGS, $P << 0.001$; Table 4). Moreover, as it appears in the same table, serum apoE was comparably high in a cohort of nephrotic patients with a different form of nephropathy, such as MN (226.35 ± 87), suggesting that the very high levels of apoE in serum is more of a general characteristic of NS than a peculiarity of a specific histopathological group. Looking at possible factors that may influence apoE serum levels in NS, we considered other lipids. As shown in Figure 5, serum apoE and cholesterol were directly correlated ($P << 0.001$), confirming our original hypothesis.

The urinary levels represented in all cases a 10,000th fraction of serum (that is, in the range of ng/mL) with significant differences between nephrotic subjects (0.088 ± 0.150 μg/mL) and those with FSGS (0.02 ± 0.06 μg/mL) versus controls (0.006 ± 0.002 μg/mL). The characterization of urinary apoE by two-dimensional electrophoresis showed the same isoforms as in this serum, therefore excluding a selective excretion.

Renal expression of apoE

Renal expression of apoE was evaluated by indirect immunofluorescence in 12 FSGS patients and was compared to 6 MN and 10 normal kidneys. The results are illustrated in Figures 6 and 7, which reported an example for every category. In all kidneys, apoE was mainly detected in blood vessels of the tubulointerstitium (Fig. 6A) and in a few tubuli (Fig. 6B); less intense, albeit evident, staining was observed in normal glomeruli (Fig. 7A). Glomeruli from patients with MN stained even more intensely (Fig. 7C), particularly in the capillary tuft. By contrast, in FSGS glomeruli (Fig. 7B) the apoE staining was completely absent, this finding being confirmed in all biopsies.

DISCUSSION

Cumulative data suggest an implication of apoE in atherosclerosis and FSGS causing at least two separate effects, one involving the apoE genotype and the other apoE levels in serum and kidney. Genetically-engineered mice lacking apoE develop hypercholesterolemia and glomerulosclerosis [8, 9], this supporting the quantitative decrease as a major effect of apoE in this setting. On the other hand, homozygous carriers of the apo ε2 genotype

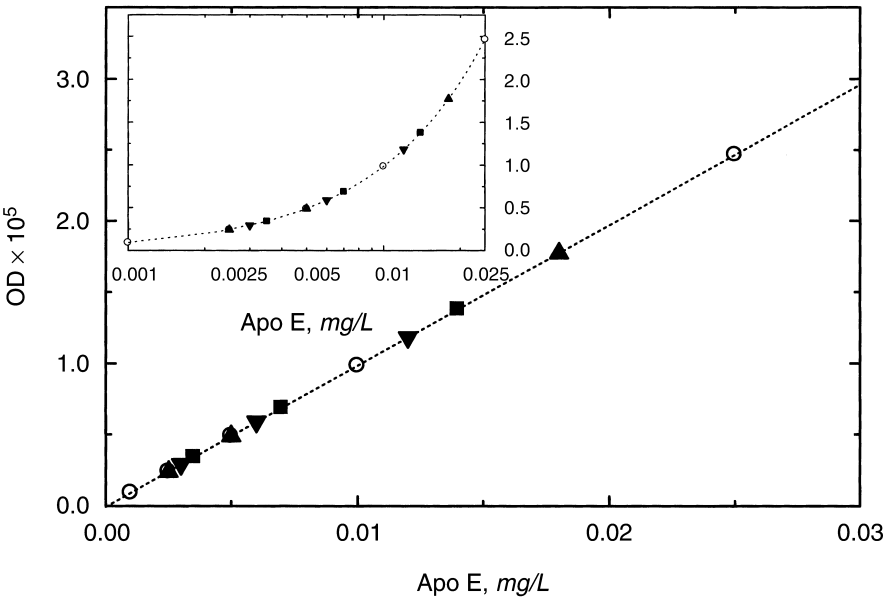

Fig. 2. Calibration curve of the dot-blot assay obtained with a purified apoE standard and with two normal sera at different dilutions. The average optical densities for chemiluminescence were plotted against the amount of apoE. The concentration of the standard was varied from 0.005 to 26 μg/mL. (Insert) The plot of a logarithmic base shows the sensitivity at very low levels. Symbols are: (○) apoE; (▲) serum 1; (▼) serum 2; (■) serum 3.

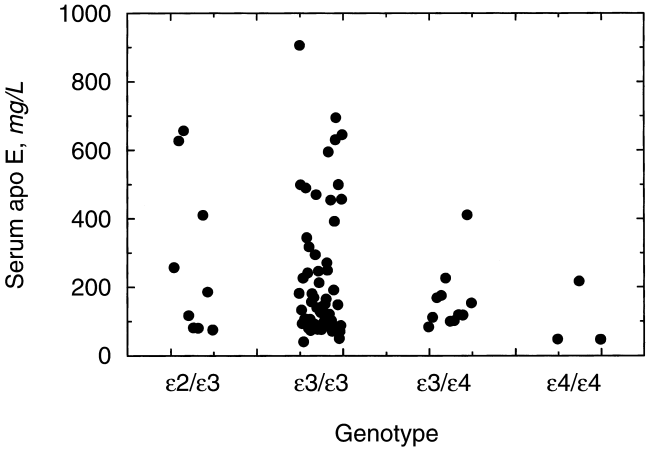

Fig. 3. Random distribution of apoE serum levels with respect to the genotype in 60 patients from whom a serum sample was obtained at different periods, unrelated from the levels of proteinuria. Serum apoE was variable within patients with the same apoE genotype. Also, those patients who had more than one determination presented variable levels despite the same genotype.

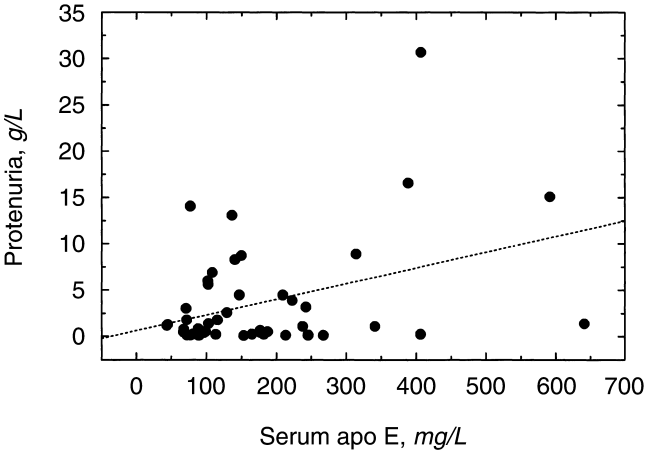

Fig. 4. Correlation between serum apoE and proteinuria in the cohort of 60 patients from a whom more than one serum sample was obtained at different phases of the disease varying from mild to overt proteinuria ($P < 0.001$). y = a + bX, where a = 0.621, b = 0.017, and $r = 0.38$.

develop hypercholesterolemia and occasionally progressive renal glomerular damage characterized by an abnormal accumulation of lipoproteins and foam cells [18]. Pathological variants of apoE such as the Sendai and others are associated also with the development of similar lesions [14–17], which supports the idea that genetic variants of apoE may be implicated in FSGS. It is noteworthy that the histological features of FSGS include the accumulation of extracellular matrix and lipids in limited areas of glomeruli and, analogous to the role of lipids in atherosclerosis, glomerular lipid accumulation has been implicated in the development of renal lesions [23]. Accordingly, hypolipemic agents confer functional and histologic protection [24–26], whereas the superimposition of alimentary hypercholesterolemia worsens the outcome of experimental FSGS in rats [27]. In humans, treatment of hyperlipemia with probucol or removal of LDL by apheresis reduces proteinuria associated with FSGS [9, 28]. The similarities between atherosclerosis and FSGS have been addressed in the past without reaching solid conclusions, but it is clear that a pathogenetic role of a molecule that is implicated in both conditions, such as apoE, is yet to be further defined.

Our data conclusively demonstrate that the predomi-

Table 4. Serum and urinary levels of apolipoprotein E in 20 children with idiopathic nephrotic syndrome (NS), 8 with focal segmental glomerulosclerosis (FSGS), 22 with membranous nephropathy (MN) and 50 normal controls

	Patient number	Serum ApoE	Urinary ApoE	Creatinine
		$\mu g/mL$		μ/g
Controls	50	44.6 ± 2.9	0.006 ± 0.002	0.007 ± 0.002
NS	20	261.9 ± 222.5[a]	0.088 ± 0.150[a]	1.173 ± 0.2
FSGS	8	180.14 ± 188.7[b]	0.020 ± 0.060[b]	0.029 ± 0.08
MN	22	226.35 ± 87.64[a]	0.044 ± 0.079[a]	0.055 ± 0.098

Urinary apolipoprotein E levels refer to the morning micturation and are expressed as μg/mL.
[a] $P << 0.001$
[b] $P < 0.001$

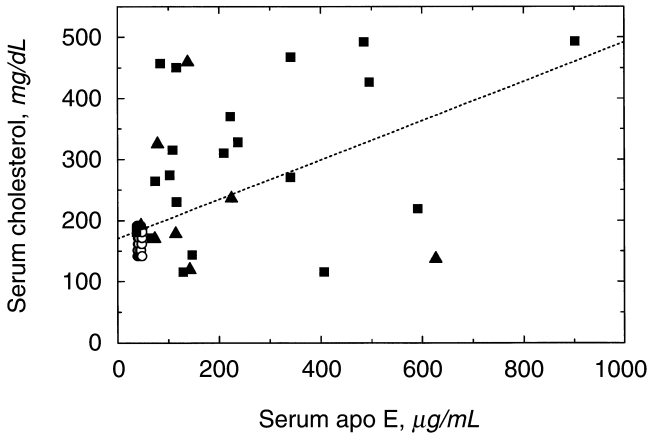

Fig. 5. Correlation between serum apoE and cholesterol in 28 children who presented nephrotic syndrome (■), eight showing the histological picture of FSGS (▲). Fifty normal controls are represented by the open circles (○; $P \ll 0.001$). y = bX, where a = 170.30, b = 0.322, and r = 0.53.

nant genotype in nephrotic patients with corticorestance is, by far, apo ε_3, and that the presence of homozygous and heterozygous carriers of the ε_2 genotype in a statistically informative population of children and adults with FSGS is only occasional. These results exclude a relevance of the apoE variants in this pathology. In this respect, we cannot confirm previous data of the literature on an association of the apo ε_2 and ε_4 alleles with FSGS in two small cohorts of patients [11] and on a higher frequency of apo ε_2 in a vast Japanese population with end-stage renal disease [13]. However, in this latter study only a small portion of patients presented proteinuria and no clear indications of the basic disease were available.

The second major finding of our study is that there is a dysregulation of apoE in nephrotic syndrome that determines very high levels in serum and in urine, while (at least in FSGS) the renal expression appears to be down-regulated. In fact, serum levels of apoE in NS is higher than in normal serum by a factor of five, this increment being correlated with hypercholesterolemia. Serum apoE levels were comparably high in a group of nephrotic patients with MN, indicating that this is a characteristic of nephrotic syndrome more than of a specific clinical group. This is an unexpected finding since apoE3, which is the most frequent variant found in our patients, is a component of HDL, whose levels in NS are unchanged or slightly decreased [3]. Obviously, we cannot exclude some pharmacological effects since almost all patients were receiving steroids. However, of the three patients not receiving steroids at the enrollment, two (patients 5 and 24) presented high apoE levels, thus suggesting that this parameter is not influenced by immunosuppression. Following the initial observation on very high apoE levels, we sought for regulatory mechanisms and excluded a direct relationship with the genotype. In a cross section of the study population, several serum samples were obtained at different times, irrespective of the disease activity, and the results on apoE levels clearly document a random distribution among patients with the same genotype and/or among the same subjects studied at different times. A relevant example is the wide variability of serum apoE in carriers of the apo $\varepsilon_3\varepsilon_3$ haplotype that present levels from the normal range up to 800 mg/L. On the other hand, in view of the strong correlation between apoE and serum cholesterol, we hypothesize that the same mechanism causing hypercholesterolemia and high LDL up-regulates apoE levels, and further studies should focus on this regulatory aspect. The significance of high apoE in the context of the severe hyperlipemia that characterizes these patients should be addressed further, since the hypocholesterolemic function of this apolipoprotein is well recognized. On the other hand, we also found that urinary levels of apoE increase in parallel with serum levels, reaching a tenfold increment in nephrotic patients. By plotting serum apoE and proteinuria a positive and high statistical correlation was found. This is in agreement with the idea that apoE, which is a low molecular weight protein of about 30 kD, is not reabsorbed at the tubular level.

The other aspect of apoE regulation in nephrotic syndrome is the low expression of apoE in glomeruli of affected patients and its complete absence in areas of sclerosis. By comparison, apoE was found to be hyperexpressed in glomeruli of other nephrotic patients with MN, suggesting that low glomerular apoE is a characteristic of FSGS. This is of particular interest, since within the glomerulus apoE inhibits mesangial cell proliferation and extracellular matrix expression, which are two mechanisms causing glomerulosclerosis. Actually, it is well known that, besides the liver, the kidney is a major source of apoE synthesis [29, 30] and RT-PCR analysis of different kidney cell types showed that mesangial cells are a major source of apoE renal expression [7]. Mice deficient in apoE have both increased proliferation and matrix

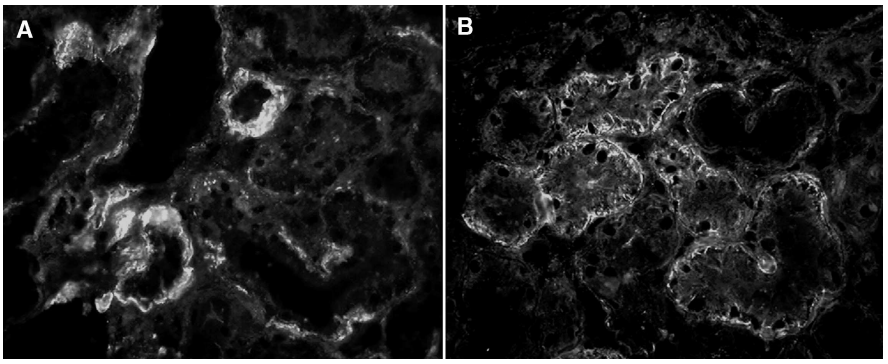

Fig. 6. ApoE expression in vascular structures of the tubulointerstitium (*A*) and in tubular cells (*B*) of a patient with nephrotic syndrome with minimal lesions.

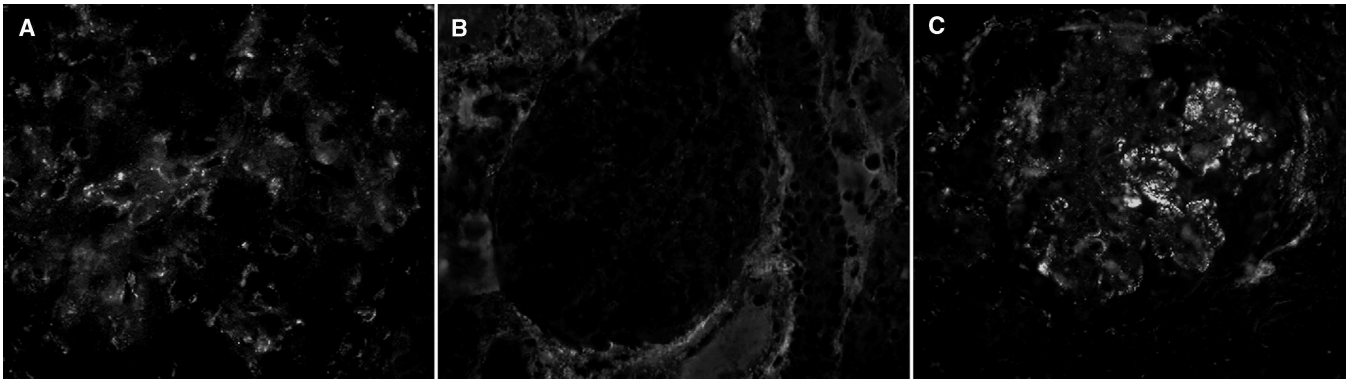

Fig. 7. ApoE expression in glomeruli deriving from (*A*) a normal kidney, (*B*) a patient with FSGS and (*C*) a patient with MN. These immunostainings are examples of what observed in several biopsies with the different pathologies (12 with FSGS, 6 with MN and 10 normal kidneys).

overproduction, two hallmarks of glomerulosclerosis. Therefore, the low protein expression in the glomeruli of patients with FSGS should negatively affect the formation of sclerotic lesions and influence progression of the disease. Other studies should address this point, as it appears to be of key importance in the process of sclerotic degeneration.

In conclusion, our study demonstrates that apoE is dysregulated in FSGS characterized by high levels in serum and urine and absence in glomeruli. Up-regulation of serum apoE is a part of the complex metabolism of lipids and also is present in other nephrotic states, but further studies are necessary to identify the intimate regulatory steps. Despite the very high serum levels, glomerular expression of apoE is down-regulated in FSGS kidneys and sclerotic areas are completely depleted of the protein. This is a specific feature of FSGS since glomerular apoE is hyperexpressed in MN. Lack of apoE-mediated antiproliferative effects and inhibition of extracellular matrix expression by mesangial cells should negatively affect glomerulosclerosis and progression of the disease. Haplotype analysis excludes predisposition to develop glomerulosclerosis given by genetic variants of the protein.

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