

Apolipoprotein E2/E5 Variants in Lipoprotein Glomerulopathy Recurred in Transplanted Kidney

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Abstract. Lipid abnormalities are associated with various disorders ranging from generalized atherosclerosis to renal diseases, including lipoprotein glomerulopathy that is characterized by glomerular lipoprotein thrombi and causes type III hyperlipoproteinemia, proteinuria, and renal failure. This study examines lipoprotein glomerulopathy, which recurred in a transplanted kidney. Molecular biologic analysis of the patient's apolipoprotein (apo) E gene demonstrated E2/E5 type variants. Immunohistochemical analysis of the diseased kidney demonstrated various lipid peroxidation-specific protein adducts, sug-

gesting a potential role of oxidative stress in this disorder. Recurrence in the transplanted kidney suggested a pathogenic role of extraglomerular humoral component(s) resulting from abnormal lipoprotein metabolism, presumably linked to apo E and other genetic or acquired factor(s). Furthermore, the finding that the patient showed pathologic abnormalities in the transplanted kidney with no clinical signs or symptoms of renal disease indicated that lipoprotein glomerular damage progresses early before any clinical manifestations.

Lipid abnormalities are associated with various disorders ranging from generalized atherosclerosis to renal diseases. The role of lipoproteins in the pathogenesis of atherosclerosis has been well demonstrated by experimental studies in animals and humans, observational evidence from population studies, and a number of controlled clinical trials (1). Abnormal lipid metabolism in patients with renal disorders is most prominent in the nephrotic syndrome, but it is also present in chronic renal failure from any cause and in patients who undergo renal transplantation. Abnormalities of lipid metabolism may also promote glomerular injury, although it is difficult to distinguish whether they are primary causes of glomerular damage or secondary consequences.

Lipoprotein glomerulopathy (LPG) is a newly recognized renal disease in which lipid deposition is limited to the kidney and is believed to cause glomerulosclerosis (2,3). This disease is characterized by glomerular lipoprotein thrombi and clinically by proteinuria and type III hyperlipoproteinemia with apolipoprotein (apo) E abnormality (4). The molecular mechanism leading to LPG is not well understood. We now report

the case of LPG with apo E variants, which recurred in transplanted kidney.

Case Report

A 42-yr-old Japanese man, who developed end-stage renal disease due to lipoprotein glomerulopathy, underwent a cadaver kidney transplant in September 1992, after 3 yr of maintenance hemodialysis. Details of his original LPG have been reported previously (5). His father died at the age of 70 due to lung cancer. His mother suffered from diabetes mellitus. His younger brother was healthy, and the family history revealed no renal disorders. The patient had no children.

The donor was a 26-yr-old man who suffered from a head trauma. The HLA was two mismatches for AB loci and none for DR loci. A biopsy specimen of the donor kidney showed no histologic abnormality at the time of transplantation. Immunosuppressive agents were prednisolone and tacrolimus (FK506). In December 1993, a renal biopsy was performed according to our protocol follow-up. Four of 44 glomeruli showed a dilation of the glomerular capillary lumen and an accumulation of lamellar-structure materials (Figure 1A). The materials were positive for Sudan III and anti-human apo E antibody. By electron microscopy, the capillary lumens were filled with granular electron-dense materials and small lipid droplets (Figure 1B). These findings were identical to his original LPG (5) and consistent with the glomerular pathology of other reported cases (3,4). Urine protein excretion was 0.3 g/d, and the urine sediment showed no abnormalities. Except for an increased plasma apo E level (20 mg/dl; normal range, 2.2 to 6.4 mg/dl), serum biochemistry and other standard laboratory examinations revealed no particular abnormalities: They included se-

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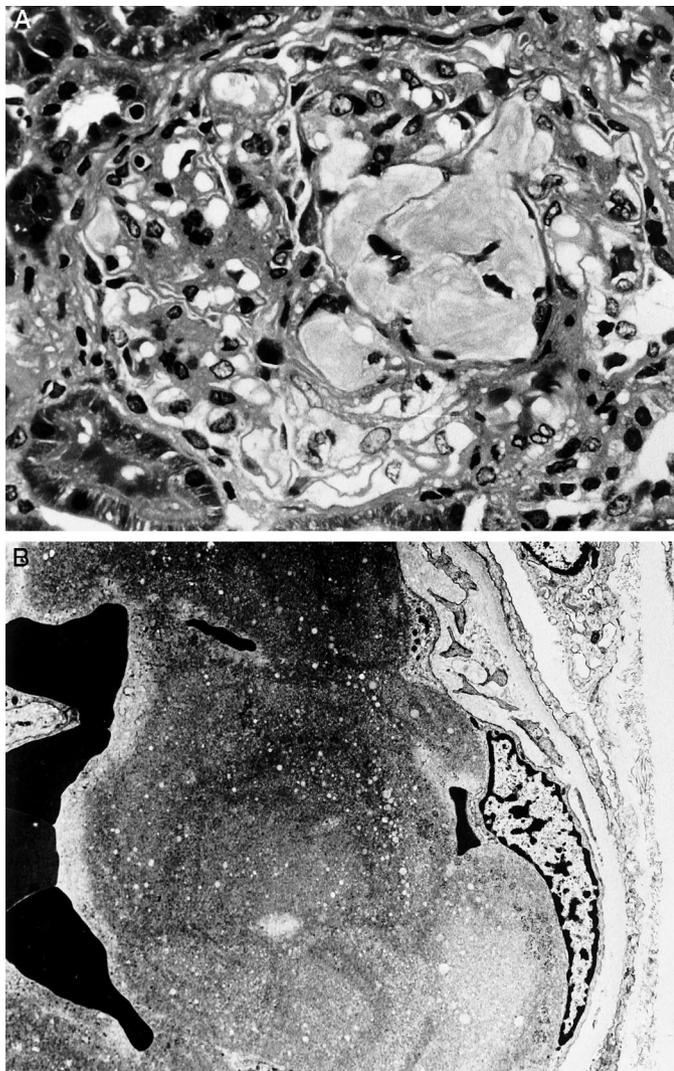


Figure 1. Light (A) and electron (B) microscopic analysis of a glomerulus from transplanted biopsy specimen. The glomerulus showed markedly dilated capillaries with accumulation of pale eosinophilic material. High magnification of the material revealed irregular layered structure with lipid vacuoles and dense granules. Flattened erythrocytes seemed to be caught between the deposits and capillary walls. Hematoxylin and eosin staining. Magnification: $\times 200$ in A; $\times 2500$ in B.

rum creatinine 1.3 mg/dl, serum albumin 4.8 g/dl, total cholesterol 217 mg/dl, triglyceride 232 mg/dl, HDL-cholesterol 67 mg/dl, apo A-I 177 mg/dl, apo A-II 39 mg/dl, apo B 111 mg/dl, apo C-II 7.8 mg/dl, and apo C-III 24.4 mg/dl.

Materials and Methods

Isoelectric Focusing

Isoelectric focusing was performed with an apo E phenotyping kit (Joukou Inc., Tokyo, Japan) using Kataoka's method (6). In brief, plasma samples were applied to polyacrylamide gels after incubation with dithiothreitol and Tween 20. Then the apo E bands were detected by immunoblotting. Identification of phenotype was accomplished by comparing the location and number of protein bands with those of controls.

DNA Sequencing of Genomic Apo E Gene

Genomic DNA was isolated from human leukocyte using Dr. GenTLE (Takara, Tokyo, Japan) according to the manufacturer's protocol. The genomic DNA was amplified using a PCR with DMSO (final 10% volume). The sets of the primers used are listed in Figure 2. Specimens were amplified for 30 cycles in a DNA thermal cycler (model 9700; Perkin Elmer Applied Biosystems, Foster City, CA). Each cycle consisted of incubation for denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 70°C for 2 min. Each PCR product was cloned into the pCRII vector using a TA cloning kit (Invitrogen, Carlsbad, CA), and more than eight clones from each PCR product were sequenced by dideoxy chain termination reaction using a DNA Autosequencer (model 377; Perkin Elmer Applied Biosystems).

Restriction Fragment Length Polymorphism Analysis

Apo E2 genotyping was carried out by DNA amplification by PCR and *HhaI* digestion, essentially as described by Hixson and Vernier (7). The amplified 240-bp DNA was digested with *HhaI*, and the DNA fragments were separated on a 5% polyacrylamide gel and stained with ethidium bromide.

Apo E5 genotyping was carried out by DNA amplification using PCR and *HphI* digestion. The PCR was performed for 30 cycles with denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 70°C for 1 min. After PCR amplification, each reaction mixture was adjusted to the concentration of 50 mM NaCl, and 5 U of *HphI* (New England Biolabs, Beverly, MA) was added directly for digestion for 2 h at 37°C. The DNA fragments were separated on an 8% polyacrylamide gel and stained by ethidium bromide.

Immunohistochemical Staining

Renal biopsy specimens were sectioned at 4 μ m and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) on ice for 15 min. After washing with PBS, the sections were blocked with 4% skim milk for 60 min at room temperature and subsequently incubated with either anti-carboxymethyllysine (CML) (8), anti-malondialdehyde (MDA)-lysine (a generous gift from Dr. Joseph L. Witztum, Department of Medicine, University of California, San Diego) (9), anti-hydroxynonenal (HNE)-protein adduct (a generous gift from Dr. Koji Uchida, Laboratory of Food and Biodynamics, Nagoya University Graduate School of Bioagricultural Sciences, Nagoya, Japan) (10), or anti-acrolein-protein adduct antibody (a generous gift from Dr. Koji Uchida) (11) at 4°C overnight. These antibodies recognize distinct structures of lipid peroxidation-protein adducts and do not cross-react with the other structures, as demonstrated previously (12,13). Nonimmune rabbit or mouse IgG was used as a negative control.

After washing with Tris-saline buffer containing 100 mM NaCl and 150 mM Tris-HCl, pH 7.5 (TBS), the sections were dehydrated through graded ethanol, incubated in methanol with 0.3% H₂O₂ at room temperature for 20 min to block endogenous peroxidase, and washed three times with TBS with 0.02% Tween 20 (Wako Pure Chemicals, Osaka, Japan). The sections for anti-CML, anti-MDA, or anti-acrolein antibody were incubated with rabbit anti-mouse IgG conjugated with peroxidase (Dako, Glostrup, Denmark), and the sections for anti-HNE antibody were incubated with swine anti-rabbit IgG conjugated with peroxidase (Dako). After washing with TBS containing Tween 20, they were developed by reactions with 3', 3'-diaminobenzidine solution containing 0.03% H₂O₂, followed by counterstaining with hematoxylin.

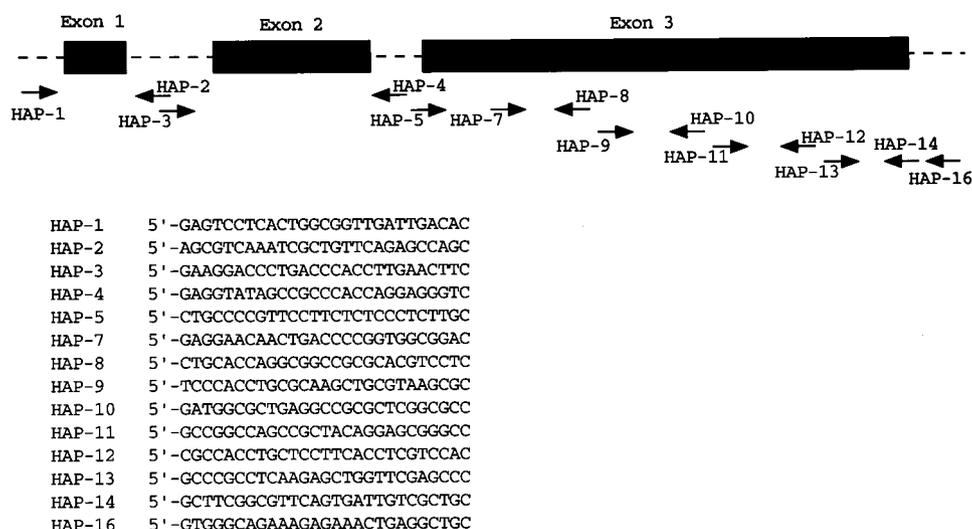


Figure 2. Locations and sequences of the primers for DNA sequencing. The apolipoprotein (apo) E gene is represented on the dotted line with relative positions of the exons shown by thick bars. Arrows represent the directions of the primers. Fourteen PCR primers ranging from 24 to 26 nucleotides in length overlapped segments to cover the genomic region corresponding to the published human apo E cDNA.

Results

Isoelectric focusing data showed that the patient's apo E phenotype was none of any combinations of E2, E3, and E4 (Figure 3). One band was clearly demonstrated between the upper band of E4 and the anode, suggesting that the patient might have apo E5. Phenotype of his mother and his brother was E2/E3 (data not shown).

To determine the genetic variant(s) causing this abnormal apo E phenotype, the patient's apo E gene was amplified using PCR reaction, and the nucleotide sequencing analysis of the genomic apo E gene was performed. The sequencing analysis revealed two variants, single substitution of lysine (AAG) for glutamic acid (GAG) at position 3 and single substitution of cysteine (TGC) for arginine (CGC) at position 158. These variants corresponded to E5 and E2, respectively. Restriction fragment length polymorphism analysis, which was performed to eliminate the sequencing error resulting from misincorporation of PCR, confirmed E2/E5 pattern (Figure 4).

To further characterize the pathologic changes in LPG, we investigated involvement of oxidative stress in this disease, as the roles of oxidative stress have been emphasized in kidney diseases and their complications. We performed immunohistochemical analysis using specific antibodies against lipid peroxidation-protein adducts. Various kinds of protein adducts formed by carbonyl amine chemistry with the autoxidation products of lipids, *e.g.*, CML (Figure 5A), HNE-adduct (Figure 5B), MDA-lysine (Figure 5C), and acrolein-adduct (Figure 5D), were identified in lipid thrombi of the patient, in colocalization with apo E (data not shown). By contrast, the antibodies for CML, HNE-adduct, MDA-lysine, and acrolein-adduct did not stain the glomeruli in normal renal tissues (12,13). No immunoreaction was observed with nonimmune rabbit or mouse IgG.

As described previously in normal and diabetic renal tissues (12,13), proximal renal tubules and peritubular vessels were

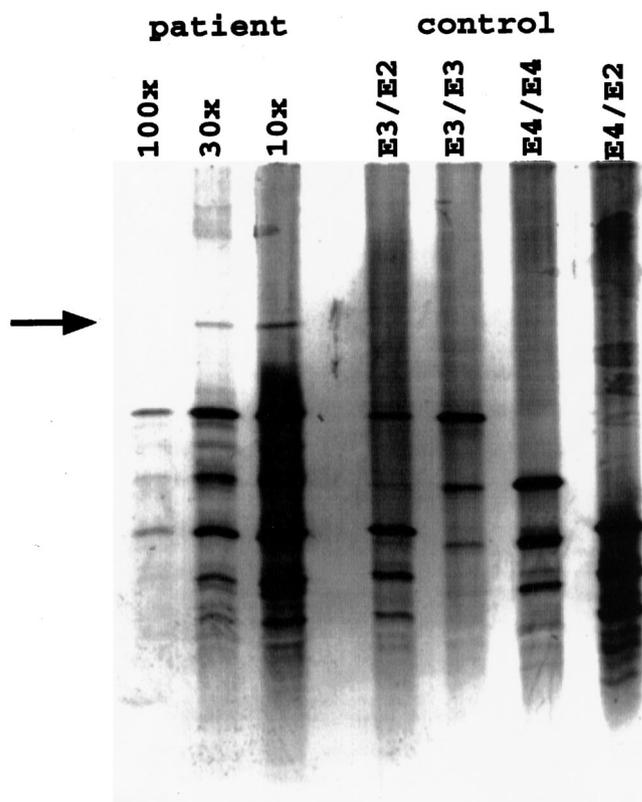


Figure 3. Phenotyping of apo E gene by isoelectric focusing. The phenotypic pattern of the patient's apo E product was distinct from any combinations of E2, E3, and E4. Note that the patient's sample demonstrated a band between the upper band of E4 and the anode (arrow), suggesting a potential E5 phenotype.

stained positive in this patient. Detection of these products within proximal renal tubules might reflect the tubular reabsorption of free-form adducts (14).

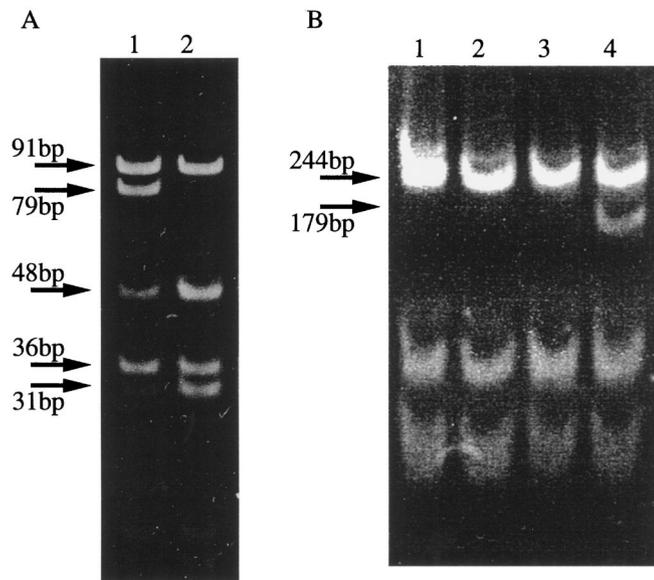


Figure 4. Restriction fragment length polymorphism analysis confirmed the E2/E5 pattern of apo E gene of the patient. (A) *HhaI* cleavage of apo E products. *HhaI* digestion of apo E of the patient contained a 79-bp fragment reflecting the absence of the restriction enzyme site at 158 Cys (apo E2). The control sample showed 48- and 31-bp fragments instead of the 79-bp band due to the cleavage at the *HhaI* site at 158 Arg (apo E3). (B) *HphI* cleavage of apo E products. Apo E of the patient digested with *HphI* demonstrated a 179-bp fragment reflecting the cleavage site at 3 Lys (apo E5). The control did not have the *HphI* cleavage site at 3 Glu (apo E3).

Discussion

LPG is a new clinical entity characterized by the presence of laminated thrombi, consisting of lipid droplets, within the lumina of dilated glomerular capillaries (reviewed in references (2 and 15)). The lipid contains apo E and apo B, which are normal components of very-LDL particles (VLDL) and their remnants, intermediate-density lipoprotein (IDL). The clinical manifestations of the disease include the insidious appearance, usually in adulthood, of the steroid-resistant nephrotic syndrome, and relatively rapid progression to glomerulosclerosis and renal failure without other systemic manifestations.

Elevation of apo E is always observed in patients with LPG (2). The major physiologic role of apo E is to mediate the interaction of lipoproteins with cell receptors, both the LDL receptor and the LDL receptor-related protein, thus modulating the *in vivo* catabolism of various lipoprotein species (16). Mature apo E is composed of 299 amino acids with a LDL receptor-binding N-terminal domain and a lipid-binding C-terminal domain. Apo E is polymorphic with three main variants, designated the wild-type apo E3, non-receptor binding apo E2, and E4 (16). These proteins differ by single amino acid substitutions at one or both of the 112 and 158 positions. Apo E has been implicated in atherosclerotic diseases by affecting plasma lipid levels (17), and type III hyperlipidemia is most frequently associated with homozygosity for apo E2. However, only approximately 5% of E2/E2 individuals develop type III hyperlipidemia, indicating that other genetic or acquired fac-

tors contribute to the etiology of the disease. Several different variants of apo E with defective binding to the LDL receptor have been identified in patients with type III hyperlipidemia (18). The apo E4 variant was shown to be a risk factor for Alzheimer's disease (19).

Investigation of the plasma lipoprotein profile in patients with LPG has revealed that many affected patients indeed have features of type III hyperlipidemia, characterized by elevated IDL and high apo E levels. Recently, Oikawa and colleagues (20) reported a substitutional variant of the proline residue for arginine residue at position 145 of apo E (apo E Sendai) in three patients from two unrelated kindred, suggesting a possible pathogenic role of dysfunctional variant apo E in LPG. The wild-type E3/E3 is rarely observed in patients with LPG. The frequency of apo E2 phenotype is extremely high among patients with LPG, and E2/E3 is most often observed (2,4). The isotype of our patient was E2/E5. E5 is a rare isotype of apo E with a high affinity for LDL receptors (21). Because his mother had E2/E3, the E2 gene is likely to have derived from his mother. We could not determine phenotype or genotype of his father, who had died due to lung cancer, and we could not determine whether the E5 gene of the patient was due to a spontaneous variant or derived from his father.

The exact pathogenesis of LPG remains to be elucidated. Although the studies described above and our study clearly demonstrated linkage between apo E variant and LPG, none has been successful in demonstrating that apo E variant is a direct cause of LPG. However, existence of lipid thrombi in glomerular capillary loops strongly suggests that apo E variant plays a role of more than just a genetic marker in this disease. We speculate that abnormal components of broad- β in this patient are preferentially taken up by mesangial cells (22), resulting in deposition of lipoprotein in subendothelial spaces and in glomerular capillaries. Since various types of apo E variants are observed in LPG patients, type III hyperlipidemia associated with these variants or the variants *per se* may be a predisposing factor of LPG, which may be caused by another unknown pathogenic factor.

Recently, important roles of oxidative stress in the pathogenesis of renal disorders and their complications have been emphasized. To elucidate roles of oxidative stress in LPG, we performed immunohistochemical analysis of the diseased kidney using specific antibodies against lipid peroxidation-protein adducts. Lipid peroxidation occurs in response to oxidative stress and forms a variety of carbonyl compounds, such as MDA, HNE, and acrolein (10,11,23). These aldehydes are highly reactive with proteins, leading to formation of CML, MDA-lysine, HNE-adduct, and acrolein adduct. These aldehydes cross-link covalently with matrix tissue proteins and alter their structure and function. They also have direct biologic effects on parenchyma cells: They cross-link cell surface proteins and stimulate cellular responses (24–26). Our immunohistochemical studies identified various kinds of lipid peroxidation-protein adducts in glomeruli of LPG. These results suggested a potential pathogenic role of oxidative stress in this disorder.

The recurrence of LPG in the cadaver transplanted kidney in

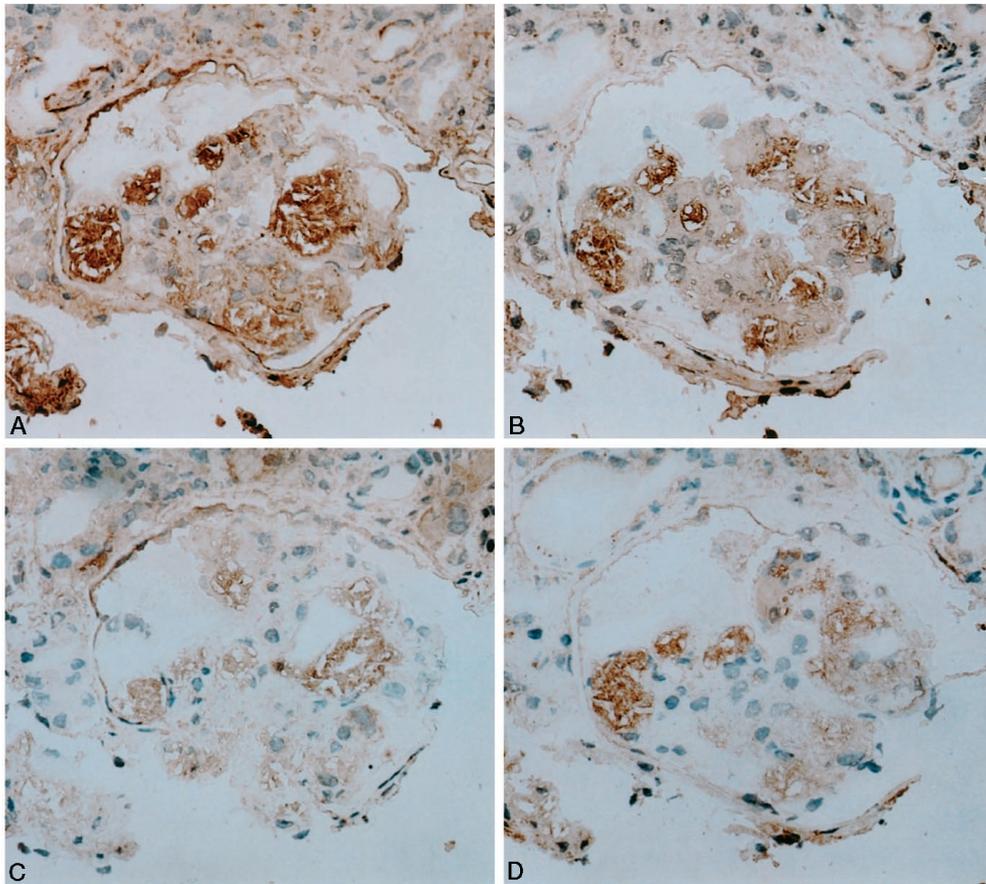


Figure 5. Various lipid peroxidation-protein adducts were detected in glomeruli of the patient. Our immunohistochemical analysis demonstrated deposition of carboxymethyllysine (A), hydroxynonenal-adduct (B), malondialdehyde-lysine (C), and acrolein-adduct (D) in lipid thrombi of the patient. Magnification, $\times 100$ in A through D.

our patient suggests clearly that the primary abnormality of this disease is not an intraglomerular factor such as an abnormality of intraglomerular lipoprotein receptor. One French case also had the recurrence of LPG in a transplanted cadaveric kidney, suggesting that LPG is caused by extrarenal factors (27).

The apparent absence of other systemic organ damage may be explained by a specific interaction between variant apo E and some undetermined elements of the glomerular capillary endothelial wall. A continuous increase of lipoprotein concentrations along the glomerular capillaries due to filtration across the capillary walls may also enhance local lipoprotein deposition and participate in determining organ specificity of LPG.

In conclusion, molecular biologic analysis of our patient's apo E gene demonstrated E2/E5 type variants. Recurrence in the transplanted kidney suggested a pathogenic role of extraglomerular humoral component(s) resulting from abnormal lipoprotein metabolism, presumably linked to apo E. Our immunohistochemical analysis suggested a pathogenic role of carbonyl stress in LPG. Furthermore, the finding that the patient showed pathologic abnormalities in transplanted kidney with no clinical signs or symptoms of renal disease indicated that lipoprotein glomerular damage progresses early before any clinical manifestations.

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