Interaction of Endothelial Cells and Triglyceride-Rich Lipoproteins With Apolipoprotein E (Arg^{25}→Cys) From a Patient With Lipoprotein Glomerulopathy

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We saw a patient with proteinuria and characteristics of lipoprotein glomerulopathy (LPG). Histologic analysis of renal biopsy showed a thrombus-like substance in the markedly dilated glomerular capillaries, which stained positive with oil red O. Increased concentration of plasma apolipoprotein E (apoE) was also noted. Those findings are consistent with the diagnostic criteria of LPG, as reported by Oikawa et al. In isoelectric focusing gel electrophoresis of apoE, a band (apoE3′) between apoE3 and E2 was observed. The patient's DNA sequence exhibited a C to G substitution in exon 3 of the apoE gene at the position of the 25th amino acid, resulting in an amino acid substitution of the arginine residue for cysteine residue. To clarify the pathophysiologic role of this mutation, we investigated the binding and the uptake of apoE3′ triglyceride-rich lipoproteins to human umbilical vein endothelial cells (HUVEC). The binding of apoE3′-triglyceride-rich lipoproteins to the cell-surface of HUVEC increased up to 30% to 50%, compared with apoE3-triglyceride-rich lipoproteins. But the uptake of apoE3′-triglyceride-rich lipoproteins into the cells was not different between them. These findings are consistent with the idea that an increase in binding of triglyceride-rich lipoproteins possessing apoE (Arg^{25}→Cys) to endothelial cells may promote deposition of lipid in the glomerular capillaries.

LIPOPROTEIN GLOMERULOPATHY (LPG) is a recently identified renal disease,[1-5] characterized by proteinuria and occasionally, nephrotic syndrome. The characteristic pathologic finding is a thombus-like substance deposited in markedly dilated glomerula capillaries. This thrombus-like substance consists of lipoproteins with apolipoproteins E (apoE) and B (apoB).[6] The presence of intermediate-density lipoproteins and a relatively high concentration of apoE is a characteristic serum lipoprotein profile.

Human apoE is a component of triglyceride-rich lipoproteins and high-density lipoproteins. Its primary function appears to be the mediation of cellular uptake of triglyceride-rich lipoproteins through both the low-density lipoprotein (LDL) receptor and LDL receptor-related protein pathways. The common genetic variations at the apoE gene locus (ε 2, ε 3, and ε 4), codes for 3 different major isoforms, are designated E2, E3, and E4, representing differing migration characteristics in isoelectric focusing (IEF).[7] The 3 common isoforms differ at polymorphic sites, with either arginine or cysteine at 112 and 158. ApoE3 and apoE4 are believed to bind equally well to lipoprotein receptors, whereas apoE2 is defective, with only approximately 1% of the binding activity of apoE3 and apoE4.[8]

Recently, Oikawa et al[9] identified an apoE variant, apoE (Arg145 Pro) Sendai, in patients with LPG. In addition, Matsunaga et al[10] reported that in another LPG patient, the sequence analysis of amplified DNA for apoE showed a C to T transition, changing the codon for residue 25 from arginine to cysteine. However, the mechanism by which this apoE variant causes LPG remains unclear.

We saw a patient with LPG, with apoE that migrated between apoE2 and apoE3 on IEF gel electrophoresis. We then analyzed the genetic structure of this apoE and the binding and the uptake activities of the triglyceride-rich lipoproteins to cultured endothelial cells.

MATERIALS AND METHODS

Characterization of a Patient With LPG

The patient was a 30-year-old male. Proteinuria and edema of the lower legs had been observed a few years ago, and at the time of presentation, the patient was suffering from nephrotic syndrome. The glomerular lesion is a thrombus like substance in the markedly dilated glomerular capillaries, which were stained positive with oil red O (data not shown). The clinical data are shown in Table 1. Serum cholesterol, triglyceride, high-density lipoprotein cholesterol, and apoE levels were 403 mg/dL, 302 mg/dL, 53 mg/dL, and 7.3 mg/dL, respectively. The patient was subjected to an analysis of the apoE phenotype, genotype, and gene mutation.

ApoE Isoform Determination

IEF was performed on polyacrylamide gel electrophoresis using a Phenotyping apoE kit (Joko Tokyo, Japan).[11] Ten microliters of serum were added to 100 μL of mixture. The apoE isoform was then separated by IEF on a 5% polyacrylamide gel containing 3 mol/L urea. The detection method included immunoblotting with goat anti-apoE anti serum as the primary antibody.

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Table 1. Initial Patient Clinical Data

Total protein (g/dL)	4.9
Serum albumin (g/dL)	3.1
Blood urea nitrogen (mg/dL)	14
Serum creatinine (mg/dL)	1.4
Serum uric acid (mg/dL)	8.0
Urine protein (g/d)	3.4
Creatinine clearance (mg/min)	31
Serum IgG (mg/dL)	268
IgA (mg/dL)	50
IgM (mg/dL)	105
C3 (mg/dL)	64
C4 (mg/dL)	20
Serum triglyceride (mg/dL)	302
Total choresterol (mg/dL)	403
HDL-choresterol (mg/dL)	53
Apolipoprotein	
A-I (mg/dL)	131
A-II (mg/dL)	18.3
B (mg/dL)	123
C-II (mg/dL)	4.3
C-III (mg/dL)	12.6
E (mg/dL)	7.6
Lecithin:cholesterol acytransferase (nmol/L/mL/h)	64

Amplification of genomic DNA by Polymerase Chain Reaction

Genomic DNA was isolated from peripheral whole blood. DNA segments were amplified using polymerase chain reaction (PCR). A volume of 25 pmol of each oligonucleotide primer was mixed in a 50-μL reaction mixture containing 10 mmol/L Tris-Hcl (pH 8.3), 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 5% dimethylsulfoxide, 200 μmol/L each of deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxycytidine triphosphate (dCTP), and deoxyguanosine triphosphate (dGTP), and 1.25 U Ex Taq DNA polymerase (Takara Syuzo, Shiga, Japan). The amplification cycle was performed on a Robo-Cycler gradient 40 Thermal Cycler (Staratagene, La Jolla, CA; Nippon Genetics, Tokyo, Japan). The mixture was incubated at 95°C for 5 minutes for denaturation and subjected to 30 cycles of amplification by primer denaturation (95°C for 1 minute), annealing (60°C for 1 minute), and extension (70°C for 2 minutes).

DNA Sequence Analysis

Genomic DNA was amplified by PCR with primers E3S and E3AS[12] and E7 and E9 as described Emi et al.[13] Direct sequencing of double-stranded DNA was performed on an ALFred DNA sequencer (Amersham Pharmacia Biotech, Piscataway, NJ), using the ThermoSequenase sequencing protocol supplied by the manufacturer (Amersham Pharmacia Biotech). A portion of the PCR product was submitted to iterative extensions on a Thermal cycler. The mixture was treated with 3minutes of denaturation at 95°C followed by 30 cycles of amplification by denaturation (95°C for 0.5 minute), primer annealing (60°C for 0.5 minute), and extension (70°C for 0.5 minute).

Restriction Enzyme Fragment Length Polymorphism Analysis

Genomic DNA was amplified by PCR with primers 3S and 3AS. To identify the point mutation of the apoE gene in codon 25, PCR products with primers 3S and 3AS were digested with the restriction enzyme *Hha*1. Fragments digested with *Hha*1 were electrophoresed on 10% polyacrylamide gel at 100 V for 120 minutes. The DNA was stained with ethidium bromide.

Uptake Assay and Binding Assay

Very–low-density lipoprotein (VLDL) prepared from the plasma of the patient was isolated by ultracentrifugation (d = 1,006) and was radioiodinated using the ^{125}I iodine monochloride method of Bilheimer et al.[14] For the binding and uptake experiments, human umbilical vein endothelial cell (HUVEC) and HepG2cell were cultured in 12-well plates. Cultured medium with 10% lipoprotein-deficient serum instead of fetal calf serum was added to the cells 24 hours before the start of the experiment.

The binding of ^{125}I-VLDL to cells was determined by incubating for 2 hours at 4°C with ^{125}I-VLDL in the presence of 50-fold excess of unlabeled VLDL as competitor. After removing the medium, the cells were washed 3 times with cold phosphate-buffered saline (PBS) containing 0.2%(wt/vol) bovine serum albumin (BSA) and subsequently with PBS without BSA. The ^{125}I-VLDL bound to the cultured cells was dissolved in 0.1 N NaOH for quantitation of the binding. An aliquot of 400 μL was counted to determine the cellular association of ^{125}I-VLDL. The binding of ^{125}I-VLDL was calculated by subtracting the value obtained in the presence of unlabeled VLDL from that in the absence of unlabeled VLDL.

The uptake of ^{125}I-VLDL to cells was determined by incubating for 8 hours at 37°C with ^{125}I-VLDL in the presence of 50-fold excess of unlabeled VLDL as competitor. After removing the medium, the cells were washed 3 times with cold PBS containing 0.2%(wt/vol) BSA and subsequently with PBS without BSA. The binding of ^{125}I-VLDL to the cultured cells was dissolved in 0.1 N NaOH for quantitation of the ^{125}I-VLDL taken up into the cells. The radioactivities in an aliquot of 400 μL were counted by gamma counter.

RESULTS

ApoE Isoform Determination

The serum apoE phenotype was determined using IEF gel electrophoresis. As shown in Fig 1, a new protein band (apoE3′), between apoE3 and E2, was found. Furthermore, under the E2 band, a faint new band was observed, corresponding to 1 sialic acid-combined form.

To confirm whether the abnormal apoE phenotype was caused by a sialic acid abnormality, the serum apoE phenotype was also analyzed after neuraminidase treatment. The band between E3 and E2 was again observed (data not shown), indicating that apoE3′ was a result of the apoE gene.

Gene Analysis

We determined the sequence of the apoE gene using direct sequencing of the PCR products. ApoE gene analysis showed a nucleotide substitution of G to C at codon 25 of the apoE gene in exon 3. As shown in Fig 2, this missense mutation denoted an amino acid substitution of cysteine residue for the arginine residue normally present at position 25 of apoE (apoE3′). No other abnormality was detected.

This mutation was confirmed by restriction enzyme digestion with *Hha*1 of PCR-amplified DNA segments, as shown in Fig 3.

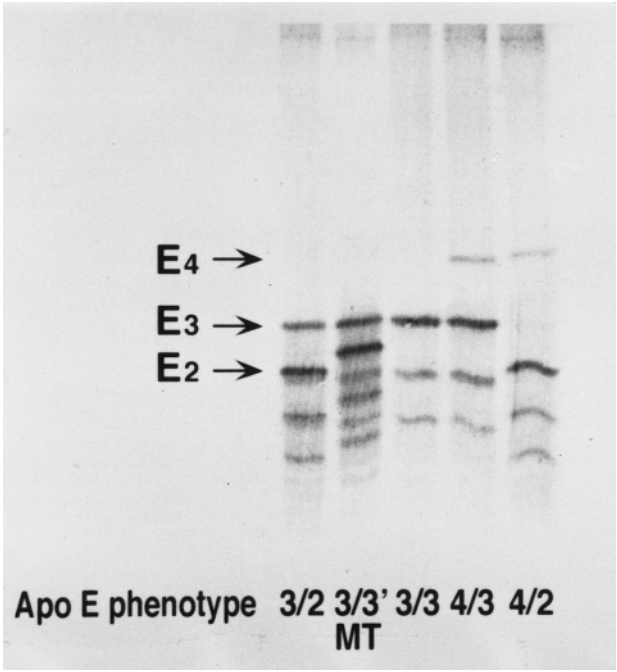

Fig 1. IEF in polyacrylamide gels and immunoblot analysis for apoE. The positions of apoE2, E3, and E4 are indicated. Lane 3/3′ corresponds to LPG patient.

Uptake Assay and Binding Assay of ^{125}I-VLDL to HUVEC and HepG2 Cells

We analyzed the receptor-binding and uptake activities of various apoE variants by in vitro assay. The cell-surface binding activity of ^{125}I-apoE3′-VLDL was higher than any other ^{125}I-apoE isoform-VLDL in both HUVEC and HepG2cells by 30% to 50% (Fig 4).

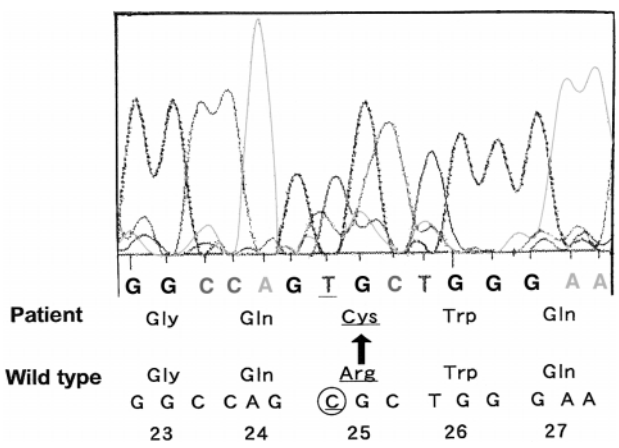

Fig 2. Sequence of genomic DNA from a LPG patient. The LPG patient has a nucleotide substitution of C to T at codon 25 of apoE gene exon 3. The normal apoE allele contained the sequence CGC coding for amino acid 25, arginine, but the patient contained TGC, coding for cysteine.

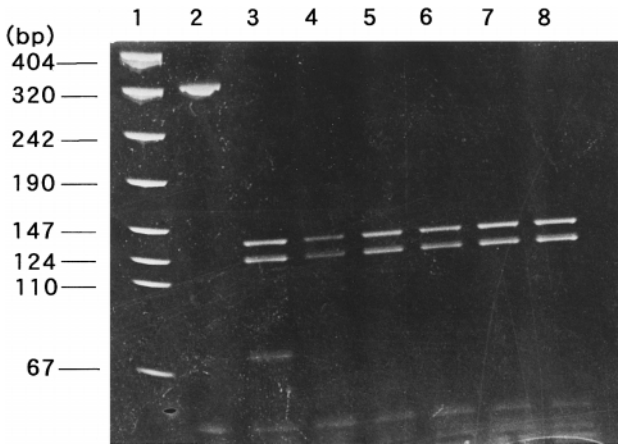

Fig 3. Polyacrylamide gel electrophoresis of amplified DNA digested with *Hha*1. Amplified DNA containing codon 25 of apoE was digested with *Hha*1, and the resulting fragments were separated on 10% polyacrylamide gel electrophoresis. Lane 1, DNA molecular weight marker VIII (Boehringer Mannheim, Mannheim, Germany); lane 2, a sample of nondigestion; lane 3, patient lane shows the 133-bp and 120-bp fragment and a 69-bp subfragment resulting from the Arg^{25}-Cys mutation; lanes 4 to 8, wild-type possesses 133-bp and 120-bp fragments only.

The uptake activity of apoE3′-VLDL into HepG2cells is apparently increased compared with those of other apoE-VLDL, but not significantly (Fig 5, left). The uptake activity of apoE3′-VLDL into HUVEC was not different from those of other apoE-VLDL (Fig 5, right).

DISCUSSION

We analyzed serum apoE in a patient with LPG and detected a new variant form, which migrated between apoE2 and E3 in IEF gel electrophoresis. The DNA sequence of the apoE gene showed a C to G substitution in exon 3 at the position of the 25th amino acid. This missense mutation represents an amino acid substitution of the arginine residue for cysteine residue, an apoE variant (Arg^{25}→Cys). This variant is coincident with apoE Kyoto.[8] However, the IEF point differs slightly. An amino acid substitution from arginine residue to cysteine residue could be expected to result in the loss of 1 charge, leading to a shift to the same point as apoE2. However, the apoE variant observed in our case migrated between apoE3 and apoE2. We digested this apoE form with neuroaminidase, but with the same result. We then sequenced the entire apoE exon. However, no other mutation was observed. We are currently unable to explain this result.

The major physiologic role of apoE is to mediate the binding of triglyceride-rich lipoproteins to LDL receptor or remnant receptor.[15,16] As for the apoE variant (Arg^{25}→Cys), Matsunaga et al[10] showed that the binding to fibroblasts was low and suggested that this low affinity might have some role in the development of glomerulonephropathy. Oikawa et al[7] suggested that apoE Sendai (Arg^{145}→Pro) represents a deformed

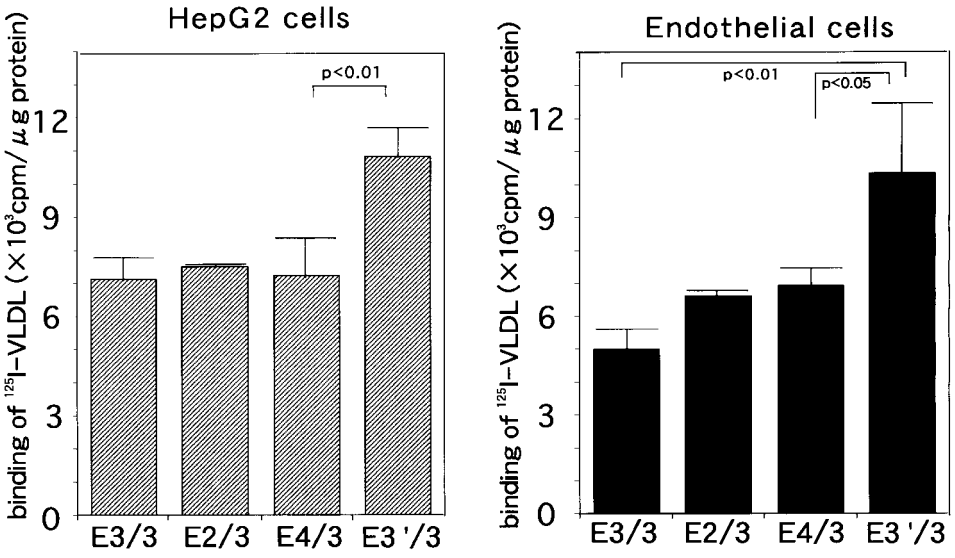

Fig 4. The cell surface binding of VLDL in various apoE isoforms. The cell surface binding activities were determined using HUVEC and HepG2cells. Cell surface binding activity at 4°C was determined by competition for various ^{125}I-labeled apoE-VLDL. Each point represents the average of triplicate measurements. The error bars correspond to SD.

protein structure, and that the deformed mutant protein may form aggregated deposits concentrated in the glomerulus. They also reported that a transgenic mouse with apoE-Sendai observed in LPG showed deposited lipids in capillary.[17] However, the mechanisms responsible for lipid deposition in the capillary lumen are unclear.

We postulate that the affinity of triglyceride-rich lipoproteins for endothelial cells is increased. We therefore studied the binding of triglyceride-rich lipoproteins from our patients to endothelial cells. We used triglyceride-rich lipoproteins, because they are the more natural forms. Endothelial cells were obtained from human umbilical vein. As shown in Fig 4, the binding of our patient's triglyceride rich-lipoproteins to endothelial cells was high compared with triglyceride rich-lipoproteins from the individuals with either apoE3 or E4. The extent of the uptake into the cells was not increased, as shown in Fig 5. The ratio of the uptake to binding activities of apoE3′-VLDL was clearly the lowest among various apoE-VLDL as shown in Fig 6 right. On the other hand, the ratio of the uptake to binding activity of apoE3′-VLDL to HepG2cells was not different from other isoforms of apoE-VLDL. One explanation for these phenomena is that the apoE3′-VLDL in our case had a high affinity for endothelial cells, but was not incorporated into the cells.

The next question is why renal capillaries were affected in LPG. It is known that glomerular capillaries work as a filter for the plasma into Bowman's cavity. Endothelial cells in the glomerular capillaries are then exposed to plasma flow mostly among various capillaries of the whole body. Therefore, a high-binding activity and low incorporation of apoE3′-lipoproteins to endothelial cells might cause lipid deposition on the

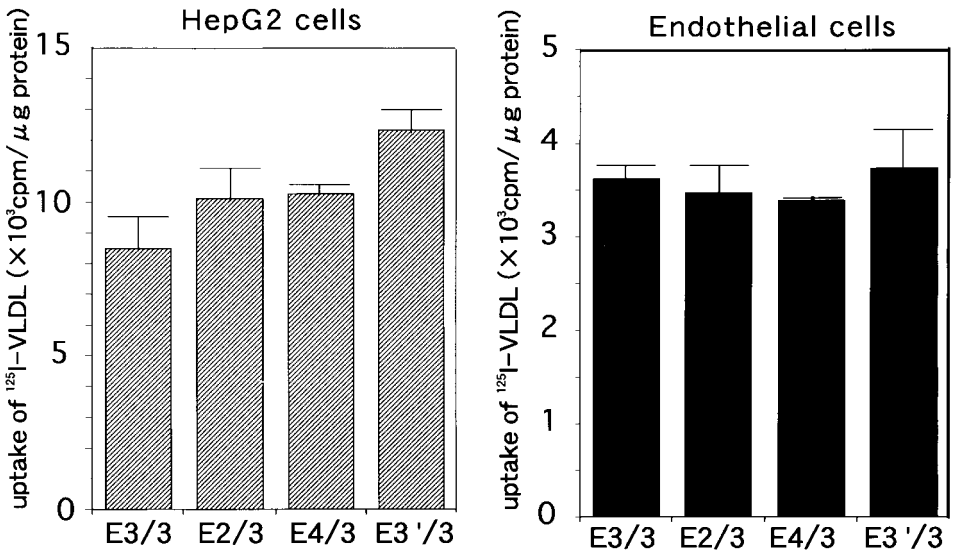

Fig 5. The uptake of VLDL in various apoE isoforms. The uptake into the cell was performed using HUVEC and HepG2 cells. Uptake into the cell at 4°C was determined by competition for various ^{125}I-labeled apoE-VLDL. Each point represents the average of triplicate measurements. The error bars correspond to SD.

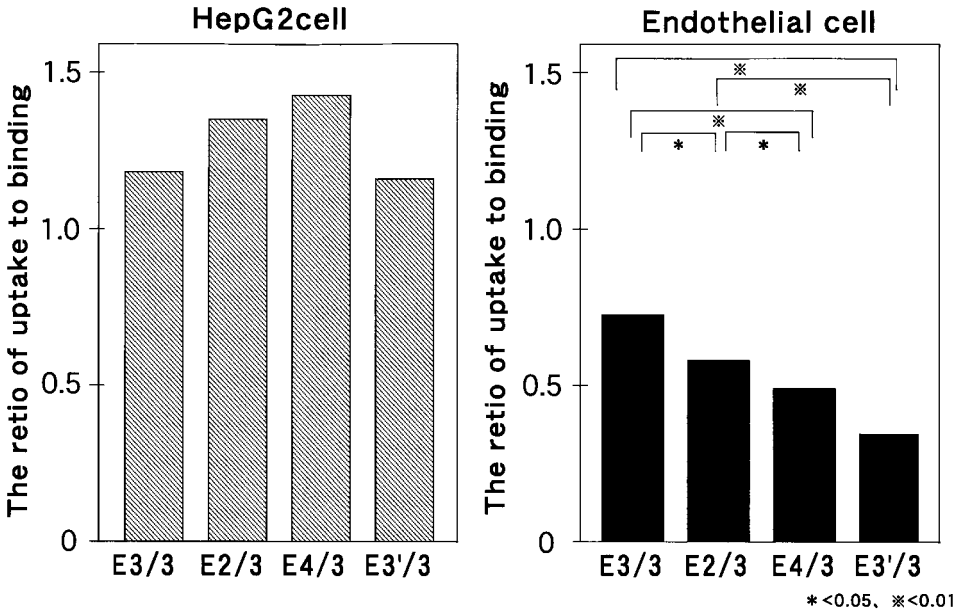

Fig 6. The ratio of binding and uptake in various apoE isoform. The cell surface binding activities and the uptake into the cell were determined by the method shown above. The ratio was shown in a relative ratio when the uptake activity was assumed to be 1.

surface of glomerular capillaries. The precise mechanisms by which thrombus-like deposition of lipids occurred in the capillaries accompanying proteinuria are still unclear. One possible factor may be oxidation of the deposited lipids, leading to injury of surrounding tissue. A recent study[18] reports that antioxidant drugs are effective in treating LPG. Further investigations are needed.

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