Virus-mediated Transduction of Apolipoprotein E (ApoE)-Sendai Develops Lipoprotein Glomerulopathy in ApoE-deficient Mice*

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Yasushi Ishigaki‡‡, Shinichi Oikawa‡, Takashi Suzuki‡, Shinichi Usui, Kenta Magorii‡, Dong-Ho Kim‡, Hiroyuki Suzuki‡, Jun Sasaki**†, Hironobu Sasanouchi, Mitsuyoshi Okazaki†, Takayoshi Toyota‡, Takao Saito**§, and Tokuo T. Yamamoto‡ ‡‡ From the ‡Tohoku University Gene Research Center, Sendai 981-8555, Japan, the §Third Department of Internal Medicine and the †Department of Pathology, Tohoku University School of Medicine, Sendai 980-8574, Japan, the ¶Laboratory of Chemistry, College of Liberal Arts and Sciences, Tokyo Medical and Dental University, Chiba 282-0827, Japan, and the **Department of Internal Medicine, Fukuoka University School of Medicine, Fukuoka 814-0180, Japan

Lipoprotein glomerulopathy (LPG) is a unique renal disease characterized by thrombus-like substances in markedly dilated glomerular capillaries, dysbetalipoproteinemia, and elevated plasma concentrations of apoE. Recent studies identified several apoE mutations in patients with LPG, including apoE2(R145P) Sendai (apoE-Sendai). Virus-mediated transduction of apoE-Sendai in apoE-deficient hypercholesterolemic mice resulted in insufficient correction of hypercholesterolemia and a marked and temporal induction of plasma triglyceride levels. In vitro binding studies showed that apoE-Sendai has a reduced affinity for the low density lipoprotein receptor, suggesting that dysbetalipoproteinemia in LPG is caused by the apoE mutation. Furthermore, histological examination revealed marked intraglomerular depositions of apoE-containing lipoproteins in mice injected with apoE-Sendai virus. These LPG-like depositions were detected 6 days after virus injection and were sustained for at least 60 days. Our results demonstrated that apoE-Sendai is an etiological cause of LPG.

Genetic abnormalities in lipoprotein metabolism are associated with various disorders ranging from classical familial hypercholesterolemia to renal diseases including lipoprotein glomerulopathy (LPG).1 LPG is a recently identified renal disease originally described in 1989 by Saito et al. (1). It is characterized by intraglomerular lipoprotein thrombi associated with dysbetalipoproteinemia and proteinuria and is frequently manifested in nephrotic syndrome and renal failure. Histological characteristics of this disease are deposition of thrombus-like substances in the markedly dilated glomerular capillaries, capillary lumina filled with dense granules, and vacuoles of lipid with lamella- and cobblestone-like formations (2). Patients with LPG have elevated concentrations of very low density lipoprotein (VLDL), intermediate density lipoprotein, and plasma apoE to levels similar to those seen in dysbetalipoproteinemia (3).

The frequency of the apoE2 phenotype in LPG patients is extremely high, and this led to the identification of a missense mutation of apoE, designated apoE2(R145P) Sendai (apoE-Sendai), in patients with LPG (4). Since the discovery of apoE-Sendai, various apoE mutations have been identified in LPG, including a missense mutation, apoE2(R25C) Kyoto (designated apoE-Kyoto) (5), and in-frame deletions, apoE3(Gln156– Gly173 → 0) (6), apoE1(His141–Leu144 → 0 or Arg142–Arg146 → 0) Tokyo (apoE-Tokyo) (7), and apoE3(His141→Lys146→0) Mae-bashi (apoE-Maebashi) (8). Excluding apoE-Kyoto, all these mutations occurred within exon 4 of the human apoE gene, which contains a cluster of basic amino acids constituting a site responsible for binding to the low density lipoprotein (LDL) receptor (9). Although these amino acid mutations may account for dysbetalipoproteinemia in patients with LPG, whether they are related to abnormal lipoprotein deposition in LPG remained unclarified.

To demonstrate that apoE-Sendai is directly related to lipoprotein deposition in LPG, we have introduced apoE-Sendai into apoE-deficient hypercholesterolemic mice using adenovirus-mediated gene transfer. Here we provide the first evidence that apoE-Sendai is an etiological cause of LPG.

MATERIALS AND METHODS

Construction of Recombinant Adenoviruses—Recombinant adenoviruses were generated by multiple ligations of restriction fragments using an adenovirus expression vector kit (Takara Shuzo, Kyoto, Japan). A control recombinant adenovirus (designated AdlacZ) containing a modified chicken β-actin promoter with a cytomegalovirus immediate-early promoter and a β-galactosidase gene was obtained from Takara Shuzo. Recombinant adenoviruses containing the entire coding regions of human apoE2, apoE3, and apoE-Sendai are designated apoE2, apoE3, and apoE-Sendai viruses, respectively. These recombinant adenoviruses were propagated in 293 cells, purified by CsCl ultracentrifugation, and stored in phosphate-buffered saline containing 10% (w/v) glycerol at ~80 °C according to the manufacturer’s instructions.

Experimental Animals—ApoE-deficient mice (10), obtained from the Jackson Laboratory (Bar Harbor, ME), were bred and housed at the Institute of Laboratory Animals of Tohoku University School of Medicine under protocols in accord with the institutional guidelines for animal experiments at Tohoku University. Animals had free access to food (4.5% fat and 0% cholesterol) and water. Age- and body weight-matched male mice (5 months of age and ~30 g, respectively) were injected with adenoviruses via the tail vein.

Lipoprotein Analysis—Blood was collected from the retro-orbital venous plexus after a 4-h fasting. Plasma total cholesterol and triglyceride levels were determined in individual mice at each time point by enzymatic assay kits (Wako Pure Chemical Co., Osaka, Japan). Immunoblotting of apoE was carried out using an ECL kit and rabbit anti-
human apoE polyclonal IgG (Sanbio-Monosan; Uden, The Netherlands). Human apoE in mouse plasma was quantified by enzyme-linked immunosorbent assay with rabbit anti-human apoE polyclonal IgG as a first antibody and a goat anti-rabbit IgG as a detecting antibody. The reaction was developed with a peroxidase substrate kit (Vector Labs, Inc.; Burlingame, CA), and absorbance was measured at 405 nm.

High Performance Liquid Chromatography (HPLC) Analysis—An improved high resolution HPLC analysis of plasma lipoprotein was performed as described (11). 20 μl of plasma was mixed with 180 μl of saline and applied to four columns of TSK Gel Lipopack XL (Tosoh, Tokyo) connected in tandem as described (11, 12). The detection of cholesterol and triglyceride in the post-column effluent was carried out by a simultaneous profiling system for lipoprotein cholesterol, triglyceride, and serum-free glycerol in an on-line system (13).

LDL Receptor Binding Assay—To overproduce recombinant apoEs, apoE-Sendai, apoE2, and apoE4 expression plasmids were constructed by multiple ligations of restriction fragments using an apoE3-expressing plasmid (a gift from Mitsubishi Kagaku Co., Yokohama, Japan) (14). The recombinant apoEs were purified by chromatography on heparin affinity and octyl-Sepharose columns. Rabbit b-VLDL (d < 1.006 g/ml) prepared from the plasma of 1% cholesterol-fed rabbits was fluorescently labeled using 1,19-dioctadecyl-3,3,3,39-tetramethylindocarbocyanine perchlorate as described (15). Dimyristoylphosphatidylcholine (DMPC) vesicles were prepared by sonication in 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 0.15 μ NaCl. Purified recombinant apoE and DMPC were mixed (1:7.5) overnight at 37 °C, and the resulting apoE-DMPC complexes (apoE-liposome complexes) were isolated by ultracentrifugation (d < 1.12) (14). Chinese hamster ovary cells stably transfected with the human LDL receptor were used for the binding assay. Cell-surface binding was carried out at 4 °C with 1,19-dioctadecyl-3,3,3,39-tetramethylindocarbocyanine perchlorate-labeled b-VLDL and apoE-DMPC complexes as competitors. The bound b-VLDL was released by suramin and quantified using a spectrofluorophotometer (15).

Histological Analysis—Kidneys were dissected after 6–60 days of virus injection and examined by light microscopy, immunohistochemistry, and electron microscopy. For light microscopy, paraffin-embedded sections were stained with Azan Mallory. Frozen sections were stained with Oil Red O or Sudan IV. For immunohistochemistry, frozen sections were stained with rabbit anti-human apoE polyclonal IgG by an indi-
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RESULTS

Plasma Expression of ApoE-Sendai in ApoE-deficient Mice—Recombinant adenoviruses containing human apoE2, apoE3, and apoE-Sendai (designated apoE2, apoE3, and apoE-Sendai viruses, respectively) were constructed and injected into apoE-deficient mice (−2 × 10^5 plaque-forming units/mouse) (10), and plasma levels of human apoE at multiple time points were determined by immunoblotting and enzyme-linked immunosorbent assay. A recombinant adenovirus (AdΔlacZ, hereafter referred to as lacZ virus) containing the β-galactosidase gene was also injected and used as a control. As shown in Fig. 1 (A and B), the levels of plasma human apoE rapidly reached maximal levels of 500–800 ng/ml 2–6 days after injection and then declined rapidly to near the baseline level 10 days after injection. There were no significant differences in the levels of plasma apoE in the mice injected with the three apoE viruses.

Plasma Lipoproteins and Lipoprotein Profiles—ApoE-deficient mice exhibit marked hypercholesterolemia with total cholesterol levels several-fold greater than those in control mice and develop severe aortic atherosclerosis (10, 16). Adenovirus-mediated expression of human apoE3 in these mice corrects hypercholesterolemia and prevents the development of atherosclerosis (17, 18). To determine the effects of apoE-Sendai on the plasma lipoprotein, we analyzed the total cholesterol and triglyceride levels and the lipoprotein profiles of the mice injected with the viruses. As shown in Fig. 2A, plasma cholesterol levels in mice injected with apoE3 virus decreased rapidly after injection, and this marked decrease was sustained for 20 days after injection and then began to increase gradually toward the baseline. ApoE-Sendai and apoE2 virus injection also decreased the levels of plasma cholesterol, but the effects were much less than with apoE3 virus. The relative reduction 4 days after injection of mice with apoE-Sendai, apoE2, and apoE3 viruses was 51, 43, and 84%, respectively. In contrast to cholesterol, the plasma triglyceride levels in mice injected with apoE-Sendai or apoE2 virus rapidly increased ~10-fold 2–6 days after injection, were sustained for several days, and then declined rapidly toward the baseline level 4–6 days after injection (Fig. 2A). Mice injected with apoE3 or control virus showed no apparent changes in plasma triglyceride levels.

HPLC analysis was carried out to determine the effects of plasma expression of apoE-Sendai in apoE-deficient mice. Plasma samples from each mouse 4 days after injection were subjected to fractionation by HPLC. Consistent with the reduction of total cholesterol levels, VLDL and intermediate density lipoprotein/LDL cholesterol levels were normalized, and high density lipoprotein cholesterol levels were reversed to the wild-type levels in mice expressing apoE3 (Fig. 2B). This reduction was much less substantial in mice expressing apoE-Sendai or apoE2. Consistent with the marked induction of triglyceride, extremely high levels of VLDL triglyceride were seen in mice injected with apoE-Sendai or apoE2 virus. Mice that received the control virus showed similar lipoprotein profiles before and after injection, indicating that these changes in lipoprotein profiles in mice injected with the apoE viruses were due to the expression of apoEs.

LDL Receptor Binding—Since the LDL receptor mediates the hepatic clearance of apoE-containing lipoproteins (19), the binding activity of apoE-Sendai to the LDL receptor was compared using an in vitro competition assay. Recombinant apoE-Sendai, apoE2, apoE3, and apoE4 were individually prepared from Escherichia coli cells carrying bacterial apoE expression plasmids, purified, and used as competitors for binding of fluorescently labeled β-VLDL to the LDL receptor. As shown in Fig. 3, the apoE-Sendai-liposome complex exhibited ~40% less inhibition of β-VLDL binding to the LDL receptor compared with the apoE3-liposome and apoE4-liposome complexes.

Histological Findings—Histological examination of mouse tissue was carried out 6–60 days after injection of viruses. Light microscopic analysis of renal sections revealed LPG-like features in the renal glomeruli of mice injected with apoE-Sendai virus. Markedly dilated capillary lumina containing mesh-like substances were seen in mice 6 days after injection of apoE-Sendai virus (Fig. 4A, panel a), whereas such abnormalities were not detected in mice injected with apoE2, apoE3, or control lacZ virus (panels b–d). In addition, typical lipoprotein droplets stained with Oil Red O were present in the capillary lumina (Fig. 4A, panel e) and were stainable with an antibody against human apoE in mice 6 days after apoE-Sendai virus injection (panel f). Quantitative examination of renal sections for lipid staining was carried out 60 days after virus injection. An average of 26% of the glomeruli were stained with Oil Red O in mice injected with apoE-Sendai virus, whereas only 7–15% were weakly positive in mice injected with apoE2, apoE3, or control lacZ virus (Fig. 4B). Furthermore, electron microscopic examination revealed capillary lumina filled with dense granules in mice injected with apoE-Sendai virus (Fig. 4C). These histological findings closely resembled those of LPG (2). Histological examination of other tissues revealed that, excluding a modest reduction of atherosclerotic lesions that was also detected in mice injected with apoE2 virus (data not shown), there were no apparent histological abnormalities in other tissues from mice injected with apoE-Sendai virus, including brain, lung, liver, testis, and adrenal gland.

DISCUSSION

In this study, we have provided direct evidence that apoE-Sendai as an etiological cause of LPG using adenovirus-mediated gene transfer in apoE-deficient mice. The data presented in this study strongly suggest that dysbetalipoproteinemia
manifested by severe hypercholesterolemia and hypertriglyceridemia in patients with LPG is caused by the apoE-Sendai mutation. As with mice expressing apoE2, a major genetic prerequisite for dysbetalipoproteinemia (20), expression of apoE-Sendai resulted in the insufficient normalization of hypercholesterolemia in apoE-deficient mice and induced high plasma levels of triglyceride. Although the mechanism of plasma triglyceride induction is currently unknown, it may be related to the hepatic uptake of plasma cholesterol by the LDL receptor. Like apoE2, apoE-Sendai exhibits a reduced binding activity for the LDL receptor, and this may lower the hepatic cholesterol pool, thereby increasing lipogenesis mediated by sterol regulatory element-binding proteins (21).

The histological findings with mice injected with apoE-Sendai virus a revealed a marked glomerular deposition of apoE-containing lipoproteins that is characteristic of LPG. These LPG-like depositions were detected 6 days after virus injection and were sustained for at least 60 days. In contrast, such abnormalities were undetectable in mice injected with apoE2 virus, suggesting that dysbetalipoproteinemia itself does not account for the glomerular deposition of lipoproteins. Then the question raised is as to why certain forms of apoE cause glo-
merular deposition in the glomerulus. The numbers of Oil Red O-positive glomeruli were calculated and divided by total glomeruli in five randomly selected sections in each mouse. Data are means ± S.E. (n = 6). Statistical differences are shown as p < 0.05 (*) and p < 0.01 (**).

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FIG. 4. Intraglomerular deposition of apoE-containing lipoproteins in apoE-deficient mice injected with apoE-Sendai virus. A, light micrographs of Azan Mallory-stained glomerular sections from mice 6 days after injection of apoE-Sendai (panel a), apoE2 (panel b), apoE3 (panel c), and lacZ (panel d) viruses and of Oil Red O-stained (panel e) and anti-human apoE antibody-immunostained (panel f) glomerular sections from mice 6 days after injection of apoE-Sendai virus. B, percentage of Oil Red O-positive glomeruli in mice 60 days after injection of apoE viruses. The percentages of Oil Red O-positive glomeruli were calculated and divided by total glomeruli in five randomly selected sections in each mouse. C, electron micrograph of a glomerulus from a mouse 60 days after apoE-Sendai virus injection. Bars in A = 10 μm.

to facilitate the development of atherosclerosis (23). Like apoB100, apoE-Sendai may have some affinity for matrix proteins or cell-surface proteins in the glomerulus, leading to the retention and accumulation of lipoproteins containing apoE-Sendai. Although the exact mechanism causing the lipoprotein deposition in the glomerulus remains unclariﬁed, the data presented here demonstrate that certain forms of apoE are an etiological cause of LPG and provide a base for the effective prevention and treatment of LPG.

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