Apolipoprotein E2 Transgenic Rabbits

MODULATION OF THE TYPE III HYPERLIPOPROTEINEMIC PHENOTYPE BY ESTROGEN AND OCCURRENCE OF SPONTANEOUS ATHEROSCLEROSIS

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Transgenic rabbits were produced that expressed high plasma levels (30–70 mg/dl) of human apolipoprotein (apo) E2(Cys-158), an apoE variant associated with the human genetic disorder type III hyperlipoproteinemia (HLP). Male transgenic rabbits fed normal chow had up to 8-fold (289 ± 148 mg/dl) and 15-fold (697 ± 452 mg/dl) increases in plasma total cholesterol and triglycerides, respectively, compared with nontransgenic males. Female transgenic rabbits had only a modest hyperlipidemia (total cholesterol, 140 ± 46 mg/dl; total triglycerides, 174 ± 66 mg/dl). Both sexes displayed the hallmarks of type III HLP: β-migrating very low density lipoproteins (β-VLDL) (intestinal and hepatic remnant lipoproteins) and significantly increased VLDL and intermediate density lipoproteins. Apolipoprotein E2-containing VLDL particles were cleared from the circulation more slowly and were more resistant to lipoprotein lipase-mediated lipolysis than normal VLDL. Only females had increased high density lipoproteins (HDL) (40%), which were shifted from typical small HDL to larger HDL1. Plasma apoE2 was predominately associated with β-VLDL in males and with HDL in females. To ascertain reasons for the phenotypic gender difference, we treated the male transgenic rabbits with 17α-ethinyl estradiol. Estrogen treatment for 10 days dramatically decreased total cholesterol (73%) and triglycerides (89%) and converted β-VLDL to pre-β-migrating VLDL. Concomitantly, lipoprotein lipase and hepatic lipase activities increased by 90%, low density lipoprotein receptor activity was stimulated significantly, apoE2 was redistributed to HDL, and HDL were converted to HDL1. Conversely, ovariection in female transgenic rabbits significantly increased total cholesterol (75%), triglycerides (117%), and β-VLDL, while decreasing lipoprotein lipase and hepatic lipase activities by 35% and redistributing apoE2 to the β-VLDL. Thus, estrogen status appears to be responsible for much of the gender difference of the lipoprotein phenotype, mainly by modulating both lipase and low density lipoprotein receptor activities. Furthermore, transgenic rabbits fed normal chow for 11 months developed fatty streaks, and some had more advanced atherosclerotic lesions, especially around the aortic arch and proximal abdominal aorta. The lesions were more extensive in males, roughly correlating with the magnitude of the hyperlipidemia. Therefore, high plasma levels of human apoE2 in transgenic rabbits result in a type III HLP phenotype, in which males have both more severe hyperlipidemia and more extensive atherosclerosis than females.

Type III hyperlipoproteinemia (HLP)1 is a genetically determined disorder of lipid metabolism in humans that is characterized by both hypercholesterolemia and hypertriglyceridemia. There is an associated accumulation of abnormal plasma lipoproteins, namely β-migrating very low density lipoproteins (β-VLDL), which are cholesterol-enriched remnant lipoproteins derived from both intestine and liver (for a review, see Ref. 1). Affected subjects are predisposed to premature atherosclerosis (1, 2). Type III HLP can be caused either by receptor binding-defective variants of apolipoprotein (apo) E (1, 3, 4), most commonly apoE2(Cys-158) (1), or by apoE deficiency (5, 6). Apolipoprotein E normally functions as a ligand for remnant uptake by lipoprotein receptors, mainly in the liver (for reviews, see Refs. 3 and 7–9). In type III HLP, defective apoE causes impaired receptor-mediated lipoprotein catabolism that leads to β-VLDL accumulation in the plasma (and thus hyperlipidemia). Type III HLP caused by apoE homozygosity is an adult-onset disease with a striking predominance in males: almost all affected women are postmenopausal, which suggests that estrogen status may modulate the expression of type III HLP in humans (1).

For the study of the pathogenesis of this disorder, models of type III HLP have been created in transgenic mice expressing apoE(Arg-112, Cys-142) (9) or apoE-Leiden (10), both of which are associated with dominant transmission of the disease in humans (11, 12), or apoE2(Cys-158) (13, 14). These transgenic mice are yielding important information for our understanding of the mechanisms of this disorder. These models have lipoprotein profiles similar to those in human type III HLP, including hypercholesterolemia, hypertriglyceridemia, and β-VLDL accumulation in plasma (9, 10) but lack the gender differences and spontaneous atherosclerosis seen in humans with the disease (15, 16). In contrast, apoE knockout mice (a model of human apoE deficiency) develop spontaneous atherosclerosis, but their lipoprotein phenotype differs from human type III HLP in that their plasma triglyceride levels are only slightly increased, and the markedly cholesterol-enriched remnant li-

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1 The abbreviations used are: HLP, hyperlipoproteinemia; apo, apolipoprotein; HDL, high density lipoprotein(s); IDL, intermediate density lipoprotein(s); LDL, low density lipoprotein(s); LPL, lipoprotein lipase; VLDL, very low density lipoprotein(s); WHHL, Watanabe heritable hyperlipidemic.
proteins that accumulate are atypical of β-VLDL in human type III HLP (17, 18). Recently, we generated an animal model of recessive type III HLP by expressing human apoE2 in transgenic mice in which both hypo- and hyperlipidemia developed, depending on the expression levels of apoE2 (13). However, because of the general resistance of mice to the development of atherosclerosis and because of some significant differences in lipoprotein metabolism between mice and humans, additional animal models of type III HLP that resemble the human disease more closely may be required to investigate the pathogenesis of this disorder and the associated susceptibility to atherosclerosis.

Rabbits have been used successfully to express several transgenes (19–22). As an experimental model, rabbits have several advantages over mice. Rabbits have higher levels of apolipoproteins than mice (23), a lipoprotein profile more like that of humans, and a pattern of hepatic apoB100 and intestinal apoB48 synthesis resembling that of humans. Like humans and unlike mice, rabbits have cholesteryl ester transfer protein, which has been reported to be elevated in rabbits (24). The larger plasma volumes in rabbits permit metabolic studies of lipoprotein subclasses and facilitate lipoprotein turnover studies. Furthermore, rabbits are very susceptible to the development of atherosclerosis, and the lesions can resemble those seen in human atherosclerosis (25, 26). For these reasons, we generated transgenic rabbits that express human apoE2 in the liver. Here, we report that high expression levels of apoE2 in transgenic rabbits lead to a type III HLP phenotype with a significant gender difference in which estrogen plays a major role. The transgenic rabbits also develop spontaneous atherosclerosis.

EXPERIMENTAL PROCEDURES

Materials—New Zealand White rabbits were purchased from Jackson Laboratories (Bar Harbor, ME). Plasmid pBSSK was purchased from Pharmacia (Uppsala, Sweden). A Superose 6 column (Pharmacia) was used on a Pharmacia fast performance liquid chromatography system. The Centricon concentration filters were from Amicon (Lexington, MA). Cholesterol and triglyceride standards were from Abbott (North Chicago, IL) and Boehringer Mannheim, respectively. The automated system for lipid analysis (Kinetic Microplate Reader) was from Molecular Devices (Menlo Park, CA). All of the reagents for lipoprotein agarose gels were from Ciba Corning (Palo Alto, CA). The ECL chemiluminescence detection kit for Western blots was purchased from Amersham (Arlington Heights, IL). All of the reagents for lipoprotein analysis were obtained from Sigma (St. Louis, MO). All of the reagents for lipoprotein analysis were obtained from Sigma (St. Louis, MO).

DNA Construct—The DNA construct used to generate transgenic rabbits was pHEG1L.Ecys158 (13). It contained the complete human apoE2 gene together with 5 kilobase pairs of its 5′-flanking sequence and 1.7 kilobase pairs of its 3′-flanking sequence, which was ligated to a 3.8-kilobase pair downstream fragment containing the hepatic control region for this gene (13, 27). In both transgenic mice and rabbits, this construct yields high expression of apoE in the liver and little expression in any other tissue (9, 19, 27).

Preparation of Transgenic Rabbits—Transgenic rabbits were prepared by microinjecting the above construct into New Zealand White rabbit embryos (19, 20). At 6 weeks of age, the resulting rabbits were weaned and maintained on a normal Chow diet. The presence of the transgene was detected by Southern blotting of 10 μg of DNA with a human apoE2 DNA probe (28) and by immunoblotting of 1 μl of plasma with a human-specific anti-apoE polyclonal antiseraum (29). In the Western blot assay, rabbit apoE and human apoE2 were semiquantitated by comparing the densitometric readings of the sample bands with those of different concentrations of purified rabbit or human apoE. All experiments were performed under protocols approved by the Committee on Animal Research, University of California, San Francisco.

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tential fat had been completely removed, the aortas were opened longitudinaly from the arch, pinned out flat on styrofoam sheets, and fixed with 3% paraformaldehyde in Dulbecco's phosphate-buffered saline for at least 24 h. After staining with Sudan IV (40) to visualize the atherosclerotic lesions, the aortas were photographed on Kodak Ektachrome Lumiere film. The slides were scanned, and large-format 24-bit color image files were produced by Edward Herderick (Laboratory of Vascular Diseases, Ohio State University, Columbus, OH). Atherosclerosis was measured by computer-based quantitative morphometry of the area of sudanophilic lesions relative to the aortic surface area. Each aorta was analyzed in five nonoverlapping regions: arch (extending from its origin at the heart to the level of the duodenum), thoracic aorta, proximal abdominal aorta, distal abdominal aorta, and terminal aorta (including the iliofemoral arteries). Morphometry was performed with an Image I/AT image analysis system (Universal Imaging Corp., West Chester, PA). Networked Silicon Graphics Iris Indigo computers linking the Gladstone Institute of Cardiovascular Disease and the Laboratory of Vascular Diseases were used to prepare probability of occurrence maps (41), which display differences in the distribution (and area) of sudanophilic lesions between experimental groups in the study. For qualitative characterization of the lesions, segments of aorta from selected animals were cross-sectioned in a cryostat and stained with Oil Red O for lipids (42) and with trichrome for collagen (43).

**Statistical Analysis—** Mean lipid levels are reported as the mean ± S.D. Data for the extent of atherosclerotic lesions in the pinned-out aortas are reported as the mean ± S.E. Differences in cholesterol levels, triglyceride levels, and the extent of atherosclerosis in the pinned-out aortas were evaluated by analysis of variance. Differences in VLDL composition and lipid changes in response to estrogen treatment and ovarectomy were assessed by t test.

**RESULTS**

**Effects of ApoE2 Expression on Plasma Lipid Levels—** Transgenic rabbits (F1) expressing high levels of apoE2 (30–70 mg/dl) were generated from one male founder whose plasma apoE2 concentration was 43 mg/dl. Table I summarizes the plasma lipid levels of transgenic rabbits analyzed.

**TABLE I**

| Human apoE | Total cholesterol | Triglycerides | HDL cholesterol |
|------------|------------------|---------------|----------------|
| Nontransgenic |                  |               |                |
| Males (n = 7) | 0                | 34 ± 5        | 44 ± 14        | 22 ± 2         |
| Females (n = 11) | 0                | 49 ± 8        | 29 ± 6         | 37 ± 7         |
| ApoE2 transgenic |                |               |                |
| Males (n = 8) | 47 ± 12          | 289 ± 148     | 697 ± 452      | 26 ± 5         |
| Females (n = 10) | 49 ± 8           | 140 ± 46°     | 174 ± 66°      | 52 ± 8°        |

*p < 0.05 versus male transgenic rabbits. Differences were evaluated by analysis of variance.

**FIG. 1.** Correlation between plasma total cholesterol (A) and triglyceride (B) levels and plasma apoE2 concentrations in 5-month-old transgenic rabbits. Plasma total cholesterol, triglyceride, and apoE2 concentrations were measured from blood obtained after an overnight fast, as described under “Experimental Procedures.”

Plasma levels (mg/dl) of lipids and human apoE2 in nontransgenic and transgenic rabbits

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**Analysis of Lipoproteins and Apolipoproteins—** Plasma lipoproteins in the transgenic rabbits were analyzed by gel filtration chromatography on a Superose 6 column (Fig. 2). Compared with a nontransgenic littermate, in which most of the cholesterol was in the HDL and LDL fractions (Fig. 2A), a male transgenic rabbit with an apoE2 plasma concentration of 63 mg/dl had dramatically increased VLDL and IDL cholesterol levels (Fig. 2B). A female rabbit with a similar plasma concentration of apoE2 (59 mg/dl) had much less severe hyperlipidemia, with lower VLDL and IDL but higher HDL (Fig. 2C), than the male transgenic rabbit (Fig. 2B). The ratio of triglyceride to cholesterol in VLDL was about 2:1 in the male transgenic rabbit and about 1:1 in the female transgenic rabbit. Another significant gender difference was that the plasma HDL elution peak was shifted from fraction 30 (typical HDL) to fraction 26 (HDL1) in the female transgenics but not in the males.

Column fractions (as shown in Fig. 2A) representing different lipoproteins were pooled, concentrated, and analyzed by agarose gel electrophoresis (Fig. 3). The nontransgenic plasma lipoproteins showed typical pre-β-migrating VLDL, a slightly slower migrating IDL, β-migrating LDL, and α-migrating HDL (Fig. 3A). In contrast, both male and female transgenic rabbits had β-migrating VLDL in both the VLDL and IDL fractions (Fig. 3B and C). The HDL1 levels were only slightly higher in male transgenic than in male nontransgenic rabbits; this difference was much more pronounced in the females. In addition, in the male transgenic rabbits, both the HDL1 and HDL fractions had particles that migrated at the α2 position (Fig. 3C). The α2-migrating HDL were present in all female transgenic rabbits analyzed.

Apolipoprotein E2 distribution among the various lipoprotein classes was analyzed for both male and female transgenic rabbits. Pooled Superose 6 lipoprotein fractions of each rabbit representing the major lipoprotein classes were separated by

demic, and there were dramatic gender differences. Male transgenic rabbits had a very significant hyperlipidemia, with about 8- and 15-fold increases in plasma total cholesterol and triglyceride levels, respectively, compared with nontransgenic males, whereas female transgenic rabbits had about 3- and 6-fold higher plasma total cholesterol and triglyceride levels, respectively, than nontransgenic females. Furthermore, apoE2 levels correlated strongly with both plasma cholesterol and triglyceride levels in males but only with plasma cholesterol levels in females (Fig. 1). Even at the higher apoE2 levels, the females were resistant to the development of severe hypertriglyceridemia and much less prone to the development of hypercholesterolemia. The HDL cholesterol levels in the female transgenic rabbits were 200% higher than in males (p < 0.05) and about 40% higher than in nontransgenic female rabbits (Table I). These results indicate that female rabbits are more resistant to apoE2-induced hyperlipidemia than males.

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12% SDS-polyacrylamide gel electrophoresis, and apoE2 was detected by anti-human apoE immunoblotting (Table II). Male transgenic rabbits had much more apoE2 in the VLDL and IDL fractions than female transgenic rabbits (49 versus 21%). Plasma concentrations of endogenous rabbit apoE were unaffected by the expression of apoE2 (3.8 ± 0.9 versus 3.5 ± 0.8 mg/dl for 9 nontransgenic and 12 transgenic rabbits, respectively), and most of the endogenous rabbit apoE was present in the HDL fractions in both males and females (data not shown).

The Superose 6-isolated β-VLDL from both male and female transgenic rabbits were characterized in more detail (Table III). The ratio of apoE2 to rabbit apoE in β-VLDL was about 7:1 in transgenic males but only 4:1 in transgenic females. The higher triglyceride levels in β-VLDL from males suggested by Fig. 2 were confirmed by determining the ratio of cholesterol (or cholesteryl esters) to triglycerides, which in males was about half of that in females. The β-VLDL from the female transgenics also had a lower apoB48:apoB100 ratio (0.15 ± 0.04 versus 0.35 ± 0.04). Negative stain electron microscopy revealed that the β-VLDL particles from males and females had similar diameters (34 ± 12 versus 37 ± 13 nm), but the particles from females had many surface protrusions that were not apparent in particles from males (data not shown). We presume these protrusions represent excess surface resulting from rapid lipolysis of the core, which would be consistent with the markedly lower triglyceride content of β-VLDL from females. This analysis demonstrates a substantial gender difference in apoE2 transgenic rabbits, with females having less severe hyperlipidemia, lower β-VLDL, lower ratios of triglycerides to total cholesterol and of apoB48 to apoB100 in the β-VLDL, and higher levels of α2-migrating HDL.

Effect of ApoE2 on VLDL Clearance and Lipolysis—To understand the mechanism of remnant accumulation in transgenic rabbits, the effect of apoE2 expression on VLDL clearance was investigated. For this study, 125I-labeled normal or apoE2 VLDL (d, 1.006 g/ml) were injected into normal rabbits, and the clearance of these particles was monitored. As shown in Fig. 4A, the presence of apoE2 dramatically slowed the clearance of transgenic VLDL (compared with normal VLDL) from the plasma of normal rabbits, suggesting that the hyperlipidemia in transgenic rabbits was at least partly caused by

**FIG. 2.** Superose 6 chromatography of 200 µl of rabbit plasma. The cholesterol and triglyceride distributions were analyzed as described under “Experimental Procedures.” Each panel shows one representative profile of several rabbits analyzed in each group. A, plasma from a nontransgenic male rabbit. B, plasma from a transgenic male rabbit (apoE2 = 63 mg/dl). C, plasma from a transgenic female rabbit (apoE2 = 59 mg/dl). TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol. The units for apoE2, total cholesterol, triglyceride, and high density lipoprotein cholesterol are mg/dl.

**FIG. 3.** Agarose gel electrophoresis of lipoprotein fractions from a nontransgenic male rabbit and from a male and a female transgenic rabbit. The VLDL are Superose 6 fractions 16–18, IDL are fractions 19–22, LDL are fractions 23–27, and HDL are fractions 28–33. The origin and migration positions of α-migrating (HDL), pre-β-migrating (VLDL), and β-migrating (LDL) lipoproteins are indicated.

| Percentage distribution of apoE2 in various lipoproteins of apoE2 transgenic rabbits |
|-------------------------------|------------------|-------------------|-------------------|-------------------|
|                               | VLDL             | IDL               | LDL + HDL         | HDL               |
| Males (n = 4)                 | 33 ± 3           | 16 ± 6            | 30 ± 3            | 21 ± 9            |
| Females (n = 4)               | 12 ± 3           | 9 ± 3             | 41 ± 2            | 39 ± 6            |
| Estrogen-treated males (n = 3)| 6 ± 4            | 5 ± 4             | 44 ± 5            | 45 ± 3            |
| Ovariectomized females (n = 3)| 27 ± 4           | 14 ± 3            | 30 ± 2            | 29 ± 2            |

Aliquots of pooled Superose 6 fractions representing the major lipoprotein classes were separated by 12% SDS-polyacrylamide gel electrophoresis and detected by anti-human apoE immunoblotting. The distribution of apoE2 in the different lipoproteins was determined by densitometric scanning.
lipase-mediated lipolysis was not affected. These results, taken together with the VLDL clearance data, suggest that the hypertriglyceridemia in transgenic rabbits is caused by apoE2-induced impairment of both VLDL clearance and lipolysis.

**Effects of Estrogen on Lipids and Lipoproteins in Male ApoE2 Transgenic Rabbits**—To investigate the potential role of sex hormones in modulating type III HLP and to explain some of the gender differences seen in apoE2 transgenic rabbits, we treated male transgenic rabbits with 17α-ethinyl estradiol. The results are summarized in Table IV. Estrogen treatment (100 μg of 17α-ethinyl estradiol/kg/day) of three male transgenic rabbits resulted in 68 and 73% decreases in total cholesterol after 5 and 10 days of treatment, respectively, without significant changes in plasma levels of apoE2. The decrease in triglycerides was even more pronounced, with 86 and 89% reductions after 5 and 10 days of treatment, respectively. Superose 6 chromatography revealed that the decrease in total cholesterol and triglycerides was due to a dramatic decrease in VLDL and IDL (β-VLDL) (data not shown). Agarose gel electrophoresis demonstrated a complete “conversion” of β-VLDL to normal pre-β-migrating VLDL in estrogen-treated male apoE2 rabbits (Fig. 5). The HDL cholesterol level increased from 24 ± 4 to 40 ± 6 mg/dl in the male transgenic rabbits treated with estrogen for 10 days (Table IV). The increased HDL were shifted from the typical HDL to the larger HDL1, which migrated at the α2 position (Fig. 5). This profile was very similar to that observed in untreated female apoE2 transgenic rabbits (compare Figs. 3C and 5). Equally interesting, the majority of the apoE2 shifted from the β-VLDL and IDL fractions (49%) to the HDL1 and HDL fractions (89%) after 10 days of estrogen treatment (Table II). Plasma concentration and lipoprotein distribution of the endogenous rabbit apoE were relatively unaffected by estrogen treatment (data not shown). Similar results for total cholesterol and triglycerides were also obtained in estrogen-treated nontransgenic rabbits but with an overall lesser response (Table IV). Superose 6 chromatography revealed that estrogen treatment of the male nontransgenic rabbits for 10 days significantly decreased VLDL, IDL, and LDL and slightly decreased HDL (data not shown), as reported previously (36).

The effect of estrogen treatment on postheparin lipolytic activity was determined in these animals. Postheparin plasma LPL and hepatic lipase activities in the male apoE2 transgenic rabbits increased by 93 and 94%, respectively, after 10 days of estrogen treatment (Table IV). The lipase values in the estrogen-treated male transgenic rabbits at 10 days were similar to those in the female apoE2 transgenic rabbits before ovariectomy (Table V).

The effect of estrogen treatment on LDL receptor activity was determined by measuring the clearance of LDL receptor antibody 9D9 from estrogen-treated and untreated male rabbits. As shown in Fig. 6, estrogen treatment for 10 days significantly stimulated clearance of the 9D9 antibody in both non-

**Table III**

| Chemical composition of Superose 6-isolated VLDL from apoE2 transgenic rabbits |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                  | TC:TG ratio     | CE:TG ratio     | E2:RE ratio     | B48:B100 ratio  |
| Nontransgenic                   |                 |                 |                 |                 |
| Males (n = 4)                   | 0.24 ± 0.03     | 0.19 ± 0.02     | 0               | 0.05 ± 0.01     |
| Females (n = 4)                 | 0.28 ± 0.05     | 0.22 ± 0.03     | 0               | 0.04 ± 0.01     |
| Transgenic                      |                 |                 |                 |                 |
| Males (n = 4)                   | 0.46 ± 0.07a    | 0.31 ± 0.05a    | 7 ± 0.5         | 0.35 ± 0.04a    |
| Females (n = 4)                 | 0.81 ± 0.05abc  | 0.55 ± 0.06abc  | 4 ± 0.3         | 0.15 ± 0.04abc  |

*p < 0.001 versus male nontransgenic rabbits.

*p < 0.001 versus female nontransgenic rabbits.

*p < 0.001 versus male transgenic rabbits.

**Fig. 4. Effects of apoE2 on VLDL clearance and VLDL lipolysis.** A, 125I-Labeled nontransgenic or apoE2 transgenic VLDL (25 μg of protein/kg of body weight) were injected intravenously into normal male rabbits, and their clearance was monitored as described under “Experimental Procedures.” Results are presented as mean ± S.D., n = 3. B, 30 μg of nontransgenic or apoE2 transgenic VLDL triglycerides was incubated with 10 μl of VLDL-depleted postheparin rabbit plasma for 30 min at 37 °C in the absence or presence of 1.2 mM NaCl. Lipase activities were calculated as described under “Experimental Procedures.” Results are presented as mean ± S.D. of determinations in four rabbits. *p < 0.001 versus nontransgenic VLDL by t test. FFA, free fatty acid.

**Fig 4B.** ApoE2 in the transgenic VLDL inhibited LPL-mediated lipolysis by 77% compared with normal VLDL, while hepatic lipase-mediated lipolysis was not affected. These results, taken together with the VLDL clearance data, suggest that the hypertriglyceridemia in transgenic rabbits is caused by apoE2-induced impairment of both VLDL clearance and lipolysis.

**Effects of Estrogen on Lipids and Lipoproteins in Male ApoE2 Transgenic Rabbits**—To investigate the potential role of sex hormones in modulating type III HLP and to explain some of the gender differences seen in apoE2 transgenic rabbits, we treated male transgenic rabbits with 17α-ethinyl estradiol. The results are summarized in Table IV. Estrogen treatment (100 μg of 17α-ethinyl estradiol/kg/day) of three male transgenic rabbits resulted in 68 and 73% decreases in total cholesterol after 5 and 10 days of treatment, respectively, without significant changes in plasma levels of apoE2. The decrease in triglycerides was even more pronounced, with 86 and 89% reductions after 5 and 10 days of treatment, respectively. Superose 6 chromatography revealed that the decrease in total cholesterol and triglycerides was due to a dramatic decrease in VLDL and IDL (β-VLDL) (data not shown). Agarose gel electrophoresis demonstrated a complete “conversion” of β-VLDL to normal pre-β-migrating VLDL in estrogen-treated male apoE2 rabbits (Fig. 5). The HDL cholesterol level increased from 24 ± 4 to 40 ± 6 mg/dl in the male transgenic rabbits treated with estrogen for 10 days (Table IV). The increased HDL were shifted from the typical HDL to the larger HDL1, which migrated at the α2 position (Fig. 5). This profile was very similar to that observed in untreated female apoE2 transgenic rabbits (compare Figs. 3C and 5). Equally interesting, the majority of the apoE2 shifted from the β-VLDL and IDL fractions (49%) to the HDL1 and HDL fractions (89%) after 10 days of estrogen treatment (Table II). Plasma concentration and lipoprotein distribution of the endogenous rabbit apoE were relatively unaffected by estrogen treatment (data not shown). Similar results for total cholesterol and triglycerides were also obtained in estrogen-treated nontransgenic rabbits but with an overall lesser response (Table IV). Superose 6 chromatography revealed that estrogen treatment of the male nontransgenic rabbits for 10 days significantly decreased VLDL, IDL, and LDL and slightly decreased HDL (data not shown), as reported previously (36).

The effect of estrogen treatment on postheparin lipolytic activity was determined in these animals. Postheparin plasma LPL and hepatic lipase activities in the male apoE2 transgenic rabbits increased by 93 and 94%, respectively, after 10 days of estrogen treatment (Table IV). The lipase values in the estrogen-treated male transgenic rabbits at 10 days were similar to those in the female apoE2 transgenic rabbits before ovariectomy (Table V).

The effect of estrogen treatment on LDL receptor activity was determined by measuring the clearance of LDL receptor antibody 9D9 from estrogen-treated and untreated male rabbits. As shown in Fig. 6, estrogen treatment for 10 days significantly stimulated clearance of the 9D9 antibody in both non-

**Table III**

| Chemical composition of Superose 6-isolated VLDL from apoE2 transgenic rabbits |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                  | TC:TG ratio     | CE:TG ratio     | E2:RE ratio     | B48:B100 ratio  |
| Nontransgenic                   |                 |                 |                 |                 |
| Males (n = 4)                   | 0.24 ± 0.03     | 0.19 ± 0.02     | 0               | 0.05 ± 0.01     |
| Females (n = 4)                 | 0.28 ± 0.05     | 0.22 ± 0.03     | 0               | 0.04 ± 0.01     |
| Transgenic                      |                 |                 |                 |                 |
| Males (n = 4)                   | 0.46 ± 0.07a    | 0.31 ± 0.05a    | 7 ± 0.5         | 0.35 ± 0.04a    |
| Females (n = 4)                 | 0.81 ± 0.05abc  | 0.55 ± 0.06abc  | 4 ± 0.3         | 0.15 ± 0.04abc  |

*p < 0.001 versus male nontransgenic rabbits.

*p < 0.001 versus female nontransgenic rabbits.

*p < 0.001 versus male transgenic rabbits.
Effects of intramuscular injection of 17α-ethinyl estradiol (100 μg/kg/day) on male apoE2 transgenic rabbits

The LPL and hepatic lipase levels were obtained from postheparin plasma. Preheparin plasma all had values <5% of postheparin samples. Differences were evaluated by t test. TC, total cholesterol; TG, triglycerides; HDL-C, HDL cholesterol; FFA, free fatty acid; HL, hepatic lipase; ND, not determined.

### Table IV

| Nontransgenic (n = 3) | ApoE2 transgenic (n = 3) |
|-----------------------|-------------------------|
| **Day 0** | **Day 5** | **Day 10** | **Day 0** | **Day 5** | **Day 10** |
| **mg/dl** | **nmol FFA/ml/min** | **mg/dl** | **nmol FFA/ml/min** | **mg/dl** | **nmol FFA/ml/min** |
| TC | 30 ± 3 | 21 ± 5 | 17 ± 3 | 235 ± 57 | 76 ± 28 | 63 ± 12 |
| TG | 61 ± 11 | 25 ± 4 | 26 ± 6 | 455 ± 152 | 63 ± 43 | 52 ± 13 |
| HDL-C | 21 ± 3 | 17 ± 2 | 14 ± 2 | 24 ± 4 | 35 ± 5 | 40 ± 6 |
| apoE2 | 0 | 0 | 38 ± 3 | 36 ± 5 | 37 ± 4 |
| LPL | 232 ± 22 | ND | 333 ± 34 | 195 ± 14 | ND | 377 ± 56 |
| HL | 44 ± 11 | ND | 69 ± 6 | 50 ± 13 | ND | 97 ± 18 |

* p < 0.001 versus the value before estrogen treatment.

**Fig. 5.** Agarose gel electrophoresis of lipoprotein fractions from an apoE2 transgenic male rabbit before and after 10 days of estrogen treatment. The VLDL are Superose 6 fractions 16–18, IDL are fractions 19–22, LDL + HDL are fractions 23–27, and HDL are fractions 28–33. The origin and migration positions of α-migrating (HDL), pre-β-migrating (VLDL), and β-migrating (IDL) lipoproteins are indicated.

transgenic and apoE2 transgenic male rabbits, suggesting probable LDL receptor up-regulation by estrogen treatment. Moreover, the apoE2 transgenic rabbits displayed a trend toward slower 9D9 clearance (lower LDL receptor expression) compared with nontransgenic rabbits (Fig. 6). Taken together, these data indicate that estrogen exerted its major lipid-lowering effect by stimulating both lipolytic and LDL receptor activities.

**Effects of Estrogen on Lipids and Lipoproteins in Female ApoE2 Transgenic Rabbits**—To determine if a deficiency of endogenous estrogen production would enhance the type III HLP phenotype, female apoE2 transgenic rabbits were ovariectomized. Removal of ovaries in three female apoE2 transgenic rabbits led to 48 and 75% increases in total cholesterol 5 and 10 days after ovariectomy, respectively, without significant changes in plasma levels of apoE2 (Table V). The increase in plasma triglycerides was even more pronounced, with 112 and 119% elevations 5 and 10 days after ovariectomy, respectively. Superose 6 chromatography demonstrated that ovariectomy in female apoE2 transgenic rabbits significantly increased VLDL and IDL (Fig. 7), both of which were β-migrating (data not shown). The ratio of triglycerides to cholesterol in the VLDL was about 2:1 (Fig. 7) and was very similar to that of the male apoE2 transgenic rabbits. The HDL cholesterol in the ovariectomized female apoE2 transgenic rabbits was slightly decreased (Table V) and shifted from larger HDL1 to smaller, more typical HDL (Fig. 7). Furthermore, the percentage of the apoE2 in the β-VLDL and IDL fractions increased from 21 to 41% 10 days after ovariectomy. This apoE2 distribution pattern was very similar to that seen in male transgenic rabbits (Table II). Both the plasma concentration and the lipoprotein distribution of the endogenous rabbit apoE were relatively unaffected by ovariectomy (data not shown). On the other hand, while plasma total cholesterol levels in the ovariectomized nontransgenic female rabbits did not change significantly, plasma triglyceride levels increased substantially 5 and 10 days after ovariectomy (Table V). Furthermore, the HDL cholesterol levels decreased from 41 ± 9 to 27 ± 8 mg/dl in the nontransgenic female rabbits 10 days after ovariectomy (Table V). The VLDL, IDL, and LDL in the nontransgenic animals increased significantly as demonstrated by Superose 6 chromatography (data not shown).

The effect of ovariectomy on postheparin plasma lipolytic activity was also determined in these animals. Both postheparin plasma LPL and hepatic lipase activities decreased by about 35% 10 days after the ovariectomy (Table V). These levels were similar to those of apoE2 transgenic male rabbits before estrogen treatment (Table IV). A similar change in postheparin plasma lipase activities was also observed in the nontransgenic female rabbits (Table V). These findings complement the results in the estrogen-treated male rabbits and indicate that estrogen status is at least partly responsible for the gender differences in the apoE2 transgenic rabbits.

**Characterization of the Atherosclerosis**—An atherosclerosis susceptibility study was carried out on both male and female apoE2 transgenics. The rabbits were maintained on a normal diet after weaning and were sacrificed at 11 months. Plasma lipid levels were measured at 4, 5, 6, 8, 10, and 11 months. The mean lipid levels of the six determinations for each rabbit are shown in Table VI. Although the lipid levels varied widely among individual rabbits, the total cholesterol and triglyceride levels were higher in four of the male transgenics than in any of the females. In contrast, HDL cholesterol levels were higher in females than in males, which resulted in a much higher ratio of total cholesterol to HDL cholesterol in males than in females.

Sudan IV staining of aortas from the transgenic rabbits revealed obvious lesions, especially in the aortic arch and the upper part of the abdominal aorta. Although male transgenics had more extensive lesions than females, the distribution of the lesions was very similar in both sexes (Fig. 8A). Quantitative analysis of lesion areas demonstrated that the nontransgenic rabbits essentially had no stained lesions, whereas the male transgenics had significant involvement of the aortic arch (24%) and the upper part of the abdominal aorta (10%) and the female transgenics had less involvement (10% aortic arch, 5% upper abdominal aorta) (Fig. 8B). Since the plasma lipid levels differed between males and females (Table VI), the gender difference in the extent of the lesions may reflect the lower total cholesterol and higher HDL cholesterol levels in females.
Endothelial cell dysfunction may be an important factor in the pathogenesis of atherosclerosis. This hypothesis is supported by studies showing that endothelial dysfunction is associated with increased risk of cardiovascular events. Furthermore, interventions that improve endothelial function, such as exercise and pharmacological therapies, have been shown to reduce cardiovascular events.

The exact mechanisms by which endothelial dysfunction contributes to atherosclerosis are not fully understood. However, it is believed that endothelial dysfunction can lead to the exposure of subendothelial matrix, which activates the inflammatory response. This, in turn, results in the recruitment of monocytes and the activation of macrophages, which contribute to the development of atherosclerotic plaques.

To better understand the role of endothelial dysfunction in atherosclerosis, further research is needed to elucidate the specific mechanisms involved. This includes identifying the factors that contribute to endothelial dysfunction and developing effective strategies to prevent and treat this condition.
and humans have a much higher cholesterol:triglyceride ratio and cholesteryl ester content than β-VLDL from transgenic mice (9, 10, 13). In human type III HLP, the VLDL cholesterol: triglyceride ratio is typically >0.3 (1). This diagnostic ratio of >0.3 was achieved in the apoE2 transgenic rabbits but not in the previously reported transgenic mouse models (9, 10, 13). This difference may be explained by the presence of cholesteryl ester transfer protein activity in rabbits (46) and its absence in mice (47). In rabbits, as in humans, cholesteryl ester transfer protein activity in rabbits (46) and its ratio of HDL to VLDL particles, presumably indicating an excess surface cholesteryl ester content than β-VLDL. Furthermore, the surface protrusions on their β-VLDL particles, presumably indicating an excess surface content resulting from rapid lipolysis of β-VLDL. Furthermore, the higher HDL levels in the female transgenic rabbits may be due to a stimulated apoAI production rate by estrogen (51) and could serve as a sink to “attract and sequester” the apoE2 from the remnant lipoproteins, leading to a decreased distribution of apoE2 in the β-VLDL and a further decrease in the already impaired clearance of remnant lipoproteins. In our studies, estrogen treatment of male apoE2 transgenic rabbits shifted the distribution of apoE2 from β-VLDL to HDL fractions and increased HDL, supporting this possible mechanism. Conversely, ovariectomy of female transgenic rabbits redistributed apoE2 from HDL to β-VLDL. The redistribution of apoE2 and the changes in HDL cholesterol were attenuated in the ovariectomized females compared with the estrogen-treated males (implying that the changes may occur at a slower rate). While this may be due primarily to the pharmacologic doses of estrogen given to the male rabbits, it is possible that the higher amount of apoE2 on female transgenic HDL may be interfering with a processing event leading to plasma HDL changes. Based on our studies so far, it seems that the apoE2 transgenic rabbits provide a good animal model for the study of other secondary factors and mechanisms that modulate the expression of type III HLP.

The status of LDL receptors in type III HLP is a matter of some interest. One hypothesis is that these receptors are upregulated in response to a “perceived” deficiency in cholesterol.

### Table VI

|                      | Total cholesterol | Triglycerides | HDL cholesterol |
|----------------------|------------------|---------------|-----------------|
| Male transgenic rabbits |
| 1                    | 117 ± 24         | 238 ± 89      | 32 ± 3          |
| 2                    | 122 ± 22         | 180 ± 49      | 28 ± 2          |
| 3                    | 186 ± 23         | 444 ± 87      | 27 ± 2          |
| 4                    | 313 ± 51         | 574 ± 171     | 25 ± 2          |
| 5                    | 346 ± 42         | 789 ± 169     | 26 ± 3          |
| 6                    | 374 ± 50         | 977 ± 122     | 25 ± 2          |
| 7                    | 425 ± 124        | 1272 ± 433    | 34 ± 5          |
| Female transgenic rabbits |
| 1                    | 100 ± 15         | 115 ± 17      | 42 ± 3          |
| 2                    | 125 ± 30         | 115 ± 59      | 51 ± 6          |
| 3                    | 192 ± 80         | 305 ± 216     | 58 ± 8          |
| 4                    | 228 ± 109        | 301 ± 140     | 51 ± 3          |
| 5                    | 230 ± 107        | 401 ± 369     | 52 ± 3          |
| 6                    | 235 ± 23         | 325 ± 98      | 57 ± 6          |
| 7                    | 237 ± 62         | 363 ± 198     | 50 ± 5          |

**Phenotypic Gender Differences in ApoE2 Transgenic Rabbits**

Both nontransgenic and apoE2 transgenic rabbit aortas were stained with Sudan IV to visualize the lesion area, as described under “Experimental Procedures.” A, pinned-out aortas from a male and a female apoE2 transgenic rabbit. B, quantitative analysis of lesion area in rabbit aortas. Values (mean ± S.E.) are presented as percentage of aortic surface covered by lipid staining. The aortic arch is defined as ending at the level of the ductus scar. NonTg, nontransgenic (n = 8); male Tg, male transgenic (n = 7); female Tg, female transgenic (n = 7). Differences were evaluated by analysis of variance, p < 0.05, transgenic rabbits (both genders) versus nontransgenic rabbits (both genders) for both aortic arch and abdominal aorta; p > 0.05, male versus female transgenic rabbits for both aortic arch and abdominal aorta.

**FIG. 8. Atherosclerotic lesions in apoE2 transgenic rabbits.**

Both nontransgenic and apoE2 transgenic rabbit aortas were stained with Sudan IV to visualize the lesion area, as described under “Experimental Procedures.” A, pinned-out aortas from a male and a female apoE2 transgenic rabbit. B, quantitative analysis of lesion area in rabbit aortas. Values (mean ± S.E.) are presented as percentage of aortic surface covered by lipid staining. The aortic arch is defined as ending at the level of the ductus scar. NonTg, nontransgenic (n = 8); male Tg, male transgenic (n = 7); female Tg, female transgenic (n = 7). Differences were evaluated by analysis of variance, p < 0.05, transgenic rabbits (both genders) versus nontransgenic rabbits (both genders) for both aortic arch and abdominal aorta; p > 0.05, male versus female transgenic rabbits for both aortic arch and abdominal aorta.
delivery because of the defective apoE2 ligand on circulating lipoproteins, resulting in low plasma LDL levels, which do occur in type III HLP (1). Another possibility is that the LDL receptors are down-regulated because they become saturated with the overwhelming levels of circulating lipoproteins (β-VLDL) in type III HLP. Our results on the plasma clearance of antibody 9D9 (Fig. 6) suggest that the latter might be the case. The 9D9 clearance data showed a trend (but did not reach statistical significance) toward expression of fewer LDL receptors in WHHL normally and require only hemizygosity for human apoE2; sclerosis in transgenic rabbits expressing high plasma levels of and apoE2 transgenic rabbits reflect a significant difference inative and/or distribution differences in lesions between WHHL involvements of the thoracic aorta. Also, there may be differ-ences in the qualitative nature of the lesions, since the lesions in this study were not particularly complex. Whether qualita-tives in the qualitative nature of the lesions, since the lesionsare due to LDL receptor deficiency (52, 53). In WHHL terol diet (25, 26). In addition, a model of spontaneous athero-rosis studies, generally by feeding with a high-fat, high-choles-three factors can be investigated. Since lipid metabolism in rabbits occur in type III HLP (1). Another possibility is that the LDL are down-regulated because they become saturated with fre-receptors are down-regulated because they become saturated with low plasma LDL levels, which do occur in type III HLP (1). cle-GO, of LDL clearance and express fewer LDL receptors in the transgenic rabbit technology. We thank Sylvia Richmond for manu-
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