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トランスジェニックマウスを用いた血漿リポタンパク代謝に対するアポリポタンパクE過剰発現の影響

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Hitoshi Shimano

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Abstract

Apolipoprotein E (apoE) has a high affinity to the cell surface low density lipoprotein (LDL) receptor. To determine the role of apoE in plasma lipoprotein metabolism, the effects of overexpression of apoE were investigated in both *in vitro* and *in vivo* studies using mouse metallothionein promotor-rat apoE genomic fusion gene. First, we made gene-transfected cell line from Chinese Hamster Ovary (CHO) cells, which constitutively produces and secretes apoE. When incubated with ^{125}I-labeled very low density lipoprotein (^{125}I-VLDL) at 37°C, the apoE secreting CHO cells took up and degraded ^{125}I-VLDL with higher affinity than control cells. The data indicated that the secreted rat apo E molecules were transferred to ^{125}I-VLDL particles, which caused a higher affinity of these particles for LDL receptors on the cells. Second, as *in vivo* studies, we created three lines of transgenic mice harboring the MTHI promotor - rat apoE fusion gene. These lines expressed rat apoE mRNA in the liver and/or in the kidney and significant amounts of rat apoE were found in plasma. Under control of metallothionein promotor, expression of the transgene was enhanced by a treatment with heavy metal ions. The highest expressor line produced rat apoE mainly in the liver, and the secreted rat apoE was almost entirely associated with plasma lipoproteins. The plasma level of rat apoE in homozygotes for the transgene was 17.4 mg / dl after zinc induction (vs 4.56 mg / dl of mouse apoE in controls). In this group, plasma cholesterol and triglycerides levels were 43%, 68% reduced as compared with controls, respectively. Heterozygotes showed decreases in both lipids to a lesser extent. Gel filtration chromatography showed that lipid reduction was mainly due to decreases in both VLDL and LDL. Especially in zinc-treated homozygotes, VLDL had almost disappeared, and a remarkable decrease in LDL and a slight decrease in high density lipoprotein were also observed, indicating that decreased plasma lipids were due to a marked reduction in lipoproteins containing apoB. Furthermore, the transgenic mice, in contrast to controls, did not develop hypercholesterolemia when fed a high cholesterol diet. From dramatic and dose-related decreases in plasma lipoproteins in transgenic mice, we concluded that apoE facilitates catabolism of plasma lipoproteins containing apoB and plays a key role in plasma lipoprotein metabolism.

Introduction

Apolipoprotein E (apo E) is mainly produced in the liver and is an important constituent of plasma lipoproteins such as chylomicron, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL) and high density lipoprotein (HDL)(for review, see ref.1). Apo E has a high affinity for LDL receptors on the surface of liver and extrahepatic cells, as well as apolipoprotein B100, and functions in metabolism of plasma lipoproteins through its interaction with LDL receptor mainly in the liver. ApoE is also thought to be a specific ligand for a putative hepatic chylomicron remnant receptor (apoE receptor) (1). ApoE is expressed in many tissues and plays a crucial role in transport and redistribution of lipids in peripheral tissues such as brain, peripheral nerve, and arterial wall (1). Several lines of evidence suggest that lipoproteins with several molecules of apoE have a higher affinity for LDL receptors than those without apoE (1-7). *In vitro* studies demonstrated that enrichment in apoE on VLDL particles increases uptake of these lipoproteins when cells were cultured with human VLDL which had been incubated with excess purified apoE or recombinant apoE (3, 8-10). Recently, we and another group have reported that intravenous administration of apoE causes the incorporation of exogenous apoE onto plasma lipoproteins, enhances clearance of VLDL, and reduces the production of LDL from VLDL, resulting in a lowered plasma cholesterol level in hypercholesterolemic rabbits (11, 12). These findings suggest that apoE functions in determining the metabolic fate of lipoproteins containing apoB100. However, *in vivo* effects of overexpression of apoE, in particular, on lipoprotein metabolism are not fully understood. To further investigate the role of apoE in lipoprotein metabolism, we attempted to produce transgenic mice which overexpress apoE in the liver (13). Recently, production of transgenic mice with human genomic apoE gene has been reported (14-16). Although these reports are informative on tissue specific regulation of the human apoE gene, the changes in plasma lipids of the transgenic mice were not significant. In contrast, we have established lines of transgenic mice with high plasma apoE levels which are overproduced in the liver under control of metallothionein promotor. In the initial process of this study, we have made cell strains

which constitutively produced and secreted apo E as an *in vitro* model, and tested the effect of apo E secretion on the cellular uptake of lipoproteins. And finally, we demonstrated the marked effect of overexpressed apoE on plasma lipoprotein profile and its level in the transgenic mice.

Materials and Methods

Materials

CHO-K cells were purchased from ATCC. Na^{125}I, α-^{32}P-dCTP, γ-^{32}P-ATP, TranSlabel (^{35}S-L-methionine), and ^{125}I-ProteinA were purchased from ICN. Ham's medium (HAM) F12, phosphate buffered saline (PBS) and new-born calf serum (NCS) were obtained from GIBCO. Geneticin (G418) and bovine serum albumin (BSA) were from Sigma. The restriction or modifying enzymes used were from Nippon Gene or Toyobo (Japan). Other reagents used were all of the first grade. BDF1 and ICR mice were purchased from Nippon Bio-supp Center.

Cell Cultures

Wild type Chinese Hamster Ovary cells (CHO-K) were maintained in HAM F12 medium containing 10 % Newborn Calf Serum (NCS) in humidified 5% CO_2 incubators at 37°C.

Construction of the fusion plasmid pMAEII

Mouse metallothionein promotor was derived from a fusion plasmid pMGH which contains the promotor of mouse metallothionein I (MTHI) and rat growth hormone gene (17). The unique XhoI site of the plasmid was converted to the BamHI site by linker ligation. The resultant plasmid was digested with BamHI and dephosphorylated with bacterial alkaline phosphatase. The plasmid pALE 31, which contains whole genomic rat apo E gene, was also digested with BamHI (18, 19). The first BamHI fragment of pALE31 covering from 11base pairs upstream from the transcription initiation site to the second BamHI site in the 3rd intron was isolated by preparative 0.9 % agarose gel electrophoresis, using a

commercial DNA purification kit (Gene Clean). These two fragments were ligated with T4 ligase to yield a plasmid which contained the MTH promotor, the first BamHI fragment of pALE31 and pBR322. The new plasmid and pALE31 were then digested with HincII and AccI. The fragments were ligated and RRI was transformed with this mixture. Using end-labelled AvaI fragments of rat apoE gene as probes, we picked up the positive clones containing the whole structure of the rat apo E gene. Plasmid DNAs were prepared on a small scale, and by analyzing their digestion patterns with some restriction enzymes, we finally selected the clone which consistently contained the MTHI promotor and the whole structure of rat genomic apo E without 5' flanking DNA and vector PUC12 derived from pALE31. The new fusion plasmid was designated pMAEII. The nucleotide sequence at the junctional site of the MTH promotor and rat apo E gene was sequenced by the dideoxy method using Sequenase sequence kit (United States Corporation, USA) and confirmed to be identical with the predicted sequence.

Transfection and Selection of CHO cells Expressing Rat Apo E

CHO-K cells were seeded at 0.8 x 10^7 cells / 10 cm dish and cultured in HAM F12 with 10% NCS for 24 h. Four hours prior to transfection, the medium was changed to 5 ml of DMEM with 10% FCS. DNA-calcium phosphate coprecipitates were prepared as follows (20): 200 µl of the solution containing 10 µg DNA of pMAEII, 2 µg DNA of pSV2Neo (21) and 250 mM $CaCl_2$ was added dropwise to the same volume of 2x HBS buffer (280 mM NaCl, 50 mM Hepes, 1.5 mM Na_2HPO_4 pH 7.0-7.1) and the mixture was kept still at room temperature for 30 min. Then the mixture was added to each medium and incubated for 6 hours. The cells were washed once with phosphate buffered saline (PBS) without $CaCl_2$ (Ca -) and shocked for 90 seconds with 15% glycerol in 1x HBS. The cells were washed with PBS and allowed to grow for an additional 48 hours in Ham F12 with 10% NCS. At this time the cells were subcultured and diluted 1: 5 in the complete medium supplemented with 600 µg / ml of G418. After 3 weeks of G418 selection, each resistant colony was isolated and cultured individually in a 24-well plate. In the confluency, the medium was changed to Ham F12 with 0.2% BSA and 50 µM $ZnSO_4$ and incubated for 24

hours. The conditioned medium were subjected to the first screening by immunoblotting for rat apo E (described below), and the positive clones which fully expressed rat apo E were selected and maintained in the complete medium with 300 μg / ml of G418. One of the highly expressive transfectants was subjected to limiting dilution in 96 well plate to confirm a single clone. A strong positive clone was obtained in the second screening and designated CHO-MAEII. A transfectant with only pSV2Neo was also acquired for control cells.

Immunoblot analysis for Rat Apo E

Anti-rat apo E rabbit serum was used for immunoblotting to detect secreted rat apo E by transfected cells. The conditioned media from CHO-MAEII or control cells cultured in HAM F12 with 0.2-4 % BSA for 24 hours were collected. Then 50 μl of sample mixed with 50 μl of 2x buffer O [the final solution containing 5.5% (v/v) ß-mercaptoethanol, 62.5 mM Tris pH 8.4, 2.3% sodium dodecyl sulfate (SDS), 10% glycerol and 0.01% blue phenol blue] was subjected to electrophoresis in sodium dodecyl sulfate polyacrylamide gel (11%) and electrophoretically transferred to nitrocellulose paper in methanol buffer containing 20% methanol, 230 mM glycine, 25 mM Tris and 0.02% SDS at 90 V of constant voltage for 5 hours according to the method of Laemmli (22). The membranes were rapidly washed with the rinse buffer containing 1% Triton X-100, 1 mM EDTA, 150 mM NaCl, 10 mM Tris (pH 7.5) and immersed in the rinse buffer containing 3% BSA at room temperature for 2 h. After two washes with the rinse buffer, the filters were incubated in the rinse buffer containing anti-rat apo E rabbit serum (0.2 μl / ml) (10) and 3% BSA at 4°C overnight. The papers were washed three times with 50 ml of the rinse buffer by shaking for 30 min and incubated in the rinse buffer containing ^{125}I-labeled Protein A (2000 cpm / ng, 0.5 μg / ml) and 3% BSA. Then the membranes were washed again three times with the rinse buffer containing 1 M NaCl, followed by air-drying and autoradiography on XAR-5 (Kodak).

Metabolic Labeling and Immunoprecipitation of Secreted Rat Apo E

The cultured cells were grown in a 3.5 cm dish in HAM F12 with 10% NCS until confluent. The cells were washed extensively with PBS and labeled in methionine free DMEM with 100 µCi of Tran35S Label containing L-^{35}S methionine and cysteine for 24 hours. The media were dialysed against 25 mM ammonium bicarbonate. One µl of antiserum and immunoprecipitation buffer were added to 50 µl of sample to give 500 µl containing 0.02% $NaPO_4$, 150 mM NaCl, 1% Triton-X 100, 1% deoxycholate and 100 µM phenylmethylsulfonyl fluoride. The mixture was rotated at 4°C overnight and immunoprecipitated with a 50 µl suspension of Pansorbin, followed by 20 min rotation at 4°C. Immunoprecipitates were centrifuged at 13000 rpm for 2 min and washed with 1 ml of immunoprecipitation buffer without deoxycholate and spun down 5 times. The final pellets were dissolved in 1x buffer O and boiled for 3 minutes. An aliquot of supernatant was subjected to electrophoresis in 11% SDS PAGE and stained with Coomassie blue, fixed with acetate and enhanced in autoradiography enhancer (EN^{3}HANCE, NEN). The gel was washed, heat-dried and autoradiographed. In another experiment, the sample was treated with neuraminidase (2 u / ml) in 0.1 M sodium acetate (pH 6.0) for 18 hours at room temperature before electrophoresis.

Lipoproteins and Binding Assay for LDL Receptor

Lipoproteins were prepared from human volunteers' plasma by sequential ultracentrifugation (23). Each fraction was floated again at the same density. Lipoproteins were labeled with Na^{125}I by a modification of the iodine monochloride method of McFarlane (24). CHO-MAEII and control cells were seeded at 1.0 x 10^5 cells / 2.2 cm well and cultured in HAM F12 with 10% NCS. In the state of sub-confluency (usually 3-4 days after seeding) the medium was changed to HAM F12 with 0.2% BSA and 50 µM $ZnSO_4$ to induce the promotor of MTHI integrated into chromosomal DNA of CHO cells and incubated for 12 hours. The labelled lipoproteins were added to the medium to yield the indicated concentrations. Binding assay at 37°C for LDL receptors was performed as described (25). The incubation period was 12 hours. Surface-bound and internalized ^{125}I-

lipoproteins were exhibited together as cell-associations. The contents of cell proteins dissolved in 0.1 N NaOH were determined by the method of Lowry (26).

Production and Analysis of Transgenic Mice A BstEII - EcoRI fragment of pMAEII for microinjection was purified by agarose electrophoresis followed by electrophoretic elution, extensive phenol - chloroform extraction and ethanol precipitation (27). A DNA solution containing approximately 1000 copies of the fragment in 10 mM Tris, pH 7.4, and 0.1 mM EDTA was injected into the male pronuclei of fertilized eggs from superovulated (C57BL / 6J x DBA / J) F1 females that had been mated to males of the same genetic background (28). The injected eggs were surgically transferred to oviducts of pseudo-pregnant ICR female mice. Offspring were weaned at 3 - 4 weeks of age, and chromosomal DNA was prepared from a small portion of the tail as described by Maniatis et al (27). Transgenic founder animals (F0) and their progeny were identified by Southern blot analysis. Tail-derived DNA (5 μg) was digested with BamHI, fractionated on 0.8% gels and transferred to nylon membranes with the 1.7-kbp BamHI fragment of rat apo E gene labelled with α^{32}P-dCTP by the random primer method (29) used as a probe.

Preparation and Analysis of Total RNA Prior to sacrifice for resection of organs, transgenic mice (F1) were given water containing 20 mM $ZnSO_4$ to induce expression of injected DNA under control of MTH promoter. Total cellular RNA was isolated by extraction with acid guanidinium thiocyanate, phenol and chloroform as described by Chomczynski and Sacchi (30). 10 μg of total RNA was subjected to electrophoresis in formaldehyde-agarose (1%) gel, and transferred to a nylon membrane. The filter was baked, pre-hybridized and hybridized as described previously (31) with the probe of 5' end-labeled synthetic oligonucleotides which was rat-specific sequence (18, 32).

Production of Homozygotes for the transgene The highest expressor of apoE, line 4-20, has been established. This line has three copies of integrated genes and the inheritance pattern of the transgene was compatible with a single autosomal integration site (13).

Homozygotes for the transgene were obtained by mating three pairs of F1 animals and confirmed by comparing quantities of the integrated gene on Southern blot (13) and plasma rat apoE in immunoblot analysis. To confirm the homozygocity for the transgene, one of six predicted female homozygotes was mated to a non-transgenic male mouse, and we found that the offspring were all positive for the integrated gene.

Analysis of Plasma Rat apolipoprotein E in Transgenic Mice Whole plasma from transgenic and non-transgenic mice were examined by immunoblot analysis. Portions (1 µl) of each sample or pooled plasma were subjected to immunoblot analysis as described above except that we used polyclonal antibody against the oligopeptide which had a rat-specific amino acid sequence Arg-Try-Arg-Arg-Pro-Ala-Pro-Arg-Asp-Arg-Ala-Glu-Ala-Leu-Ser corresponding to 188 - 202 amino acid residues of rat apoE (18). Anti rat-specific antibody was affinity-purified from antiserum. This antibody does not cross-react with mouse apoE (13). In some experiments, plasma rat apoE levels from the transgene in the transgenic mice were determined by immunoblot assay using this anti-rat specific antibody with purified rat apoE as the standard. For determination of mouse apoE level in the non-transgenic controls, anti-rat apoE polyclonal antibody which is cross reactive with mouse apoE was used with purified mouse apoE as the standard.

Animal Treatment Procedure Homozygotes and Heterozygotes of line 4-20, and non-transgenic littermates (12 weeks of age) were used in the present study. There were no significant differences in body weights among controls and transgenic mice. To induce expression of the transgene, animals were given water containing 20 mM $ZnSO_4$ for one week. Fasting (12 hours from 21:00 to 9:00) blood was drawn from retroorbital plexus into a tube containing 0.1% EDTA before and after zinc treatment. Plasma was separated by centrifugation at 4°C. For high cholesterol feeding, transgenic mice and control animals (n=5) were given chow containing 1% cholesterol and 0.5% deoxycholate, and water supplemented with 20 mM $ZnSO_4$ for ten days.

Other Analyses The concentrations of cholesterol and triglycerides in plasma were determined enzymatically (33, 34). ApoB levels in pooled plasma (6 homozygotes, 19 heterozygotes, 16 controls) were determined by an isopropanol precipitation method (35).

Gel Filtration Chromatography Plasma samples (10 µl) were injected into a combined column system composed of TSK G3000SW (Tosoh Co, Tokyo) and Superose 6 HR (Pharmacia) in sequence, and chromatographed, using 0.15 M NaOH, 10 mM TrisHCl (pH 7.4) and 0.01% EDTA at a rate of 0.4 ml / min. The effluent was monitored at 280 nm and was combined with an enzymatic reagent for measurement of cholesterol or triglycerides at a rate of 0.25 ml / min. Enzymatic reaction was performed in a Teflon tube in a temperature controlled bath. The final effluent was monitored at 550 nm. This high performance liquid chromatography (HPLC) system showed elution patterns with three distinguished elution positions of the major lipoprotein classes: VLDL, LDL and HDL, in the monitoring of protein, cholesterol and triglycerides (see Fig. 3). Consistency of the data from this system with those from sequential ultracentrifugal floatation technique was confirmed in human plasma samples (36). For determination of distribution of apoE among plasma lipoproteins, pooled plasma (100 µl) from heterozygotes or controls (n=10) was applied to the columns. Without enzymatic reaction, the effluent was fractionated. Each fraction (1 ml) was monitored at 280 nm and peak fractions corresponding to VLDL, LDL, and HDL were collected. An aliquot (100 µl) was subjected to immunoblot assays using the anti-rat apoE polyclonal antibody.

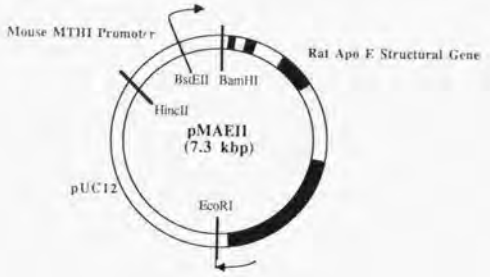
Plasma Kinetics of Retinyl Palmitate Chow was drawn during the experiment. Aqueous retinyl palmitate (2 mg) was administered orally through a gatric cannula. Blood samples were drawn at indicated hours after administration. Plasma levels of retinyl palmitate were determined by fluorometry on HPLC after extraction by hexane.

Results

ApoE Secreting Cells; CHO-MAEII

We constructed the plasmid containing the rat genomic apo E gene whose 5' flanking region is replaced with the promotor of MTHI as shown in Fig. 1. In this construction, the predicted transcription of rat apo E gene under control of the MTH promotor must be initiated at the site 11 bp upstream from the authentic transcription initiation site of rat apo E gene under control of the intrinsic promotor. To determine whether pMAEII was functional in spite of this extra sequence on transcription and translation, CHO cells were co-transfected with MAEII and pSV2Neo, which contains neomycin-resistant gene for stable expression (21). Stable transfectants were selected on the basis of resistance to G418 in the culture medium. We acquired a cell line that stably expressed rat apo E and designated it CHO-MAEII. Figure. 2a shows the representative results of immunoblotting of the conditioned media from transfectants for rat apo E. Northern blot analysis showed that rat apo E messenger RNA was transcribed constitutively and increased further by metal induction (Fig.2b). ^{35}S-labeled rat apo E secreted in the medium was immunoprecipitated and analyzed on SDS-PAGE. As shown in Fig. 2c bands were detected in doublet at 34 kd and 38 kd, and a single 34 kd band was found after treatment with neuraminidase. This suggests that rat apo E particles secreted from CHO-MAEII are almost sialylated. Using the immunoblot assay with purified rat apoE as standards, the concentrations of apo E in the medium after 12 and 24 hours of incubation were estimated as 1.07 and 1.28 μg / ml (0.99 and 1.18 μg / 10^4 cells), respectively.

a.



b.

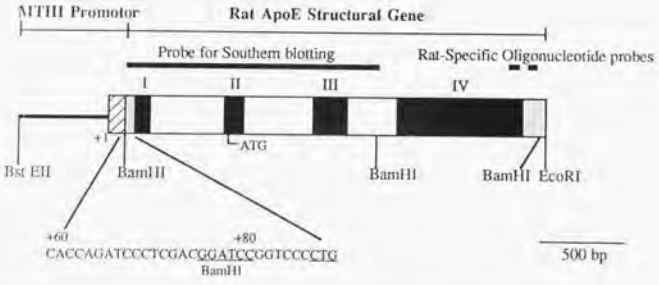


Figure 1. DNA constructs for transfection and microinjection

A fusion plasmid, pMAEII (a), containing the promotor region of MTHI, the rat apoE structural gene, and vector pUC12 was constructed as described under Methods. A BstEII-EcoRI fragment (a: indicated by the arrow, b) was used for microinjection. The construct of the injected DNA was as follows: metal regulatory region of MTHI promoter (bold line), 5'untranslated sequences of MTHI (striped box), BamHI linker sequences, 5'flanking region (densely stippled box), exons (solid boxes with Roman numerals), introns (open boxes), and 3'untranslated region containing polyA signals of the apoE gene (sparsely stippled box). The transcription initiation site of rat apoE gene under the intrinsic promoter is indicated as underlined CTG in the sequencing and the translation initiation site is indicated as ATG in the exon II. The probes used are indicated for Southern blotting and Northern blotting.

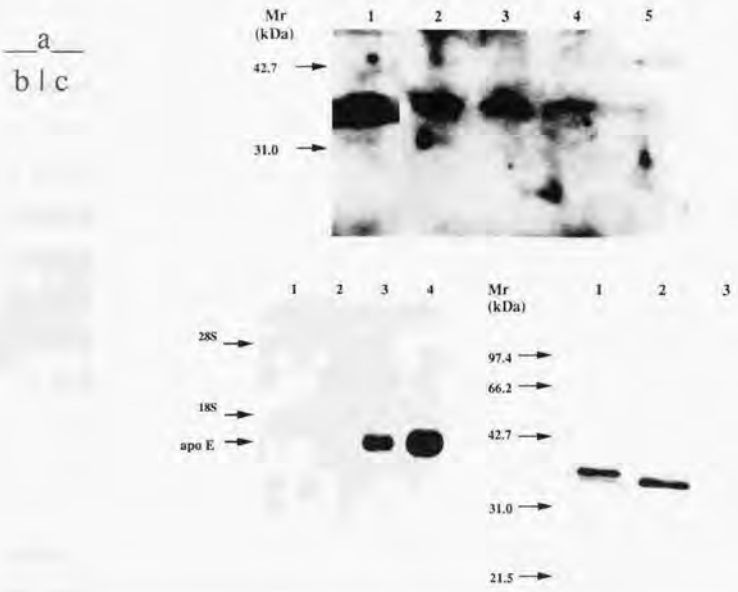


Fig. 2a. Immunoblot analysis of apo E in the conditioned medium by CHO-MAEII

For screening transfected CHO cells highly expressive of rat apoE, conditioned media (50 µl / each lane) from transfected cell clones were subjected to immunoblot as described in Materials and Methods. Molecular weight standards are indicated. 1: rat serum (1 µl) as positive control of rat apoE, 2-4: samples from GM418 resistant transfected CHO cell clones, 5: sample from untransfected CHO-K cells as negative control. The authentic rat apo E protein was detected on lane 1 at 34 kDa with heavy chain of immunoglobulin G at 50 kDa. The strong positive clone on lane 2 was designated CHO-MAEII.

b. Detection of rat apo E mRNA from CHO-MAEII by Northern blot analysis

Total cellular RNA (10 µg) from the transfectants was subjected to Northern blotting as described in Materials and Methods. 1: control cells, 2: control cells treated with zinc ion (50 µM, for one day), 3: CHO-MAEII uninduced, 4: CHO-MAEII zinc-induced. 18S and 28S ribosomes are indicated.

c. Immunoprecipitation of apo E secreted by CHO-MAEII

Cells were incubated for 24 hours in the presence of L-^{35}S methionine with methionine free medium in 2.2 cm dishes. Cell-conditioned medium was mixed with anti-rat apo E serum, followed by the addition of ProteinA. Immunoprecipitates were subjected to 10 % SDS-polyacrylamide gel electrophoresis and autoradiographed. 1: CHO-MAEII conditioned medium, 2: CHO-MAEII conditioned medium treated with neuraminidase, 3: control cell conditioned medium

Binding assays of ^{125}I-VLDL were performed for CHO-MAEII and control cells in which only pSV2Neo was transfected. Prior to the assay, the cells were pre-incubated in the medium containing 0.2% BSA for 12 hours and the indicated concentrations of ^{125}I-VLDL were added, followed by another 12 hours of incubation. Saturation kinetics of cell-association and degradation at 37°C were shown in Fig. 3. CHO-MAEII took up and degraded more ^{125}I-VLDL than control cells, especially at low concentrations of VLDL (1-10 μg / ml)(Fig. 3a). The difference was less prominent at high concentrations, and no difference was found at the concentration of saturation (50 μg / ml)(Fig. 3b). Scatchard plot showed that the affinity of VLDL for CHO-MAEII was about 4.2- fold greater than that for control cells in the uptake (Fig. 3b inset, the mean Kd's from three independent experiments are 32.47 μg / ml vs 7.77 μg / ml). Meanwhile, there were no differences in maximal uptake and degradation. The data indicated increased affinity but not increased capacity of VLDL for LDL receptors in interactions between VLDL and LDL receptors on the transfected cells. This suggested that rat apo E molecules secreted by CHO-MAEII were transferred onto ^{125}I-VLDL in the medium and that the increase in the number of apo E molecules on VLDL caused the increase in affinity for LDL receptors on the cells. To confirm the transfer of secreted rat apo E onto human VLDL in the medium, the following experiment was performed. CHO-MAEII was incubated with human VLDL and the VLDL in the conditioned medium was reisolated by ultracentrifugation and subjected to immunoblot analysis (Fig.4). Using anti-rat apoE antibody, rat apoE was detected in VLDL reisolated from CHO-MAEII, while that from control cells contained only minimal apoE detectable probably due to cross reactivity with human apoE. Anti-human apoE antibody detected a greater amount of apoE in VLDL from CHO-MAEII than that from control cells, suggesting additional rat apoE onto human VLDL particles from CHO-MAEII.

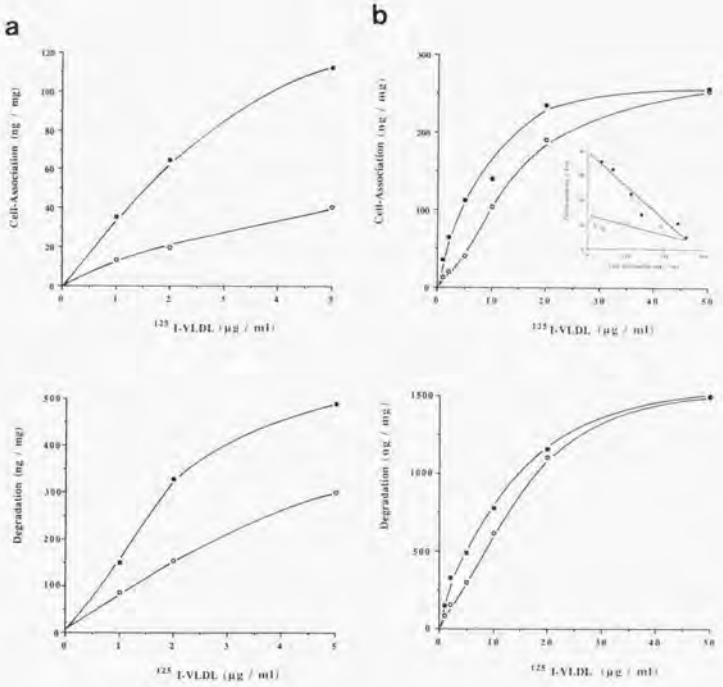


Fig. 3a,b. Saturation kinetics in cell-association and degradation of ^{125}I-VLDL in CHO-MAEII

CHO-MAEII and control cells subconfluent were preincubated in HAM with 0.2% BSA and 50 µM ZnSO4 for 12 hours, and the indicated concentrations of ^{125}I-VLDL (specific activity = 150 -400 cpm / ng) in the presence or absence of 50-fold unlabeled VLDL were added. After another 12 hours of incubation at 37°C, the medium and the cells were harvested to measure cell-associated and degraded amounts of ^{125}I-VLDL. Saturation curves of cell-association (upper) and degradation (lower) in CHO-MAEII (closed circles) and control cells (open circles) at low concentrations (1-5 µg / ml, a) and high concentrations (1-50 µg / ml, b) are shown. The values are expressed as means of the values in the absence of excess cold minus those in the presence in triplicate assays from the representative results of three independent experiments. Scatchard plots from cell-association are shown in the inset. Kd values from three independent experiments (where three different lots of VLDL were used) were CHO-MAEII: 7.67, 8.97 and 6.67, and control cells: 50.45, 32.2 and 14.8, respectively (µg / ml).

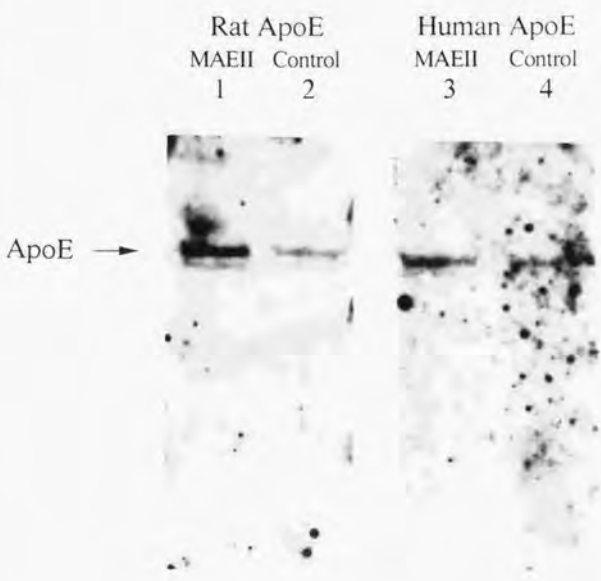


Fig. 4. Immunoblot of apoE on reisolated VLDL in the conditioned medium by CHO-MAEII

CHO-MAEII (1, 3) and control cells (2, 4) were preincubated in HAM containing 0.2% BSA and 50 μM ZnSO4 for 12 hours. Human VLDL (10 μg / ml) was added and the cells were further incubated for 12 hours. The conditioned medium was ultracentrifuged and refloated VLDL was subjected to immunoblotting using anti-rat apoE antibody (1, 2) or anti-human apoE antibody (3, 4) as described in Materials and Methods.

Production of ApoE Transgenic Mice

The DNA fragment used for microinjection (Fig. 1) contains the metal responsive element of MTHI and no elements for steroid or inflammation factors (37). Out of 68 newborn mice from 300 injected eggs transferred, eight founder transgenic mice were identified by Southern blot analysis of tail tip-derived DNA using the internal rat 1.7-kbp BamHI fragment as a probe (Fig. 5). This probe hybridized comparatively weakly with mouse apoE gene, however, the band detected in the control mouse genomic DNA digested with BamHI was different in size from that in rat DNA (mouse 1.2 kbp vs rat 1.7 kbp).

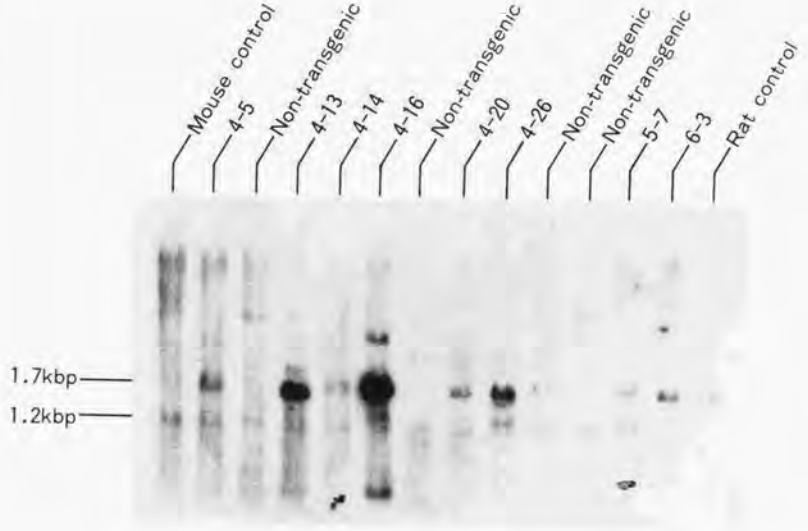


Figure 5. Southern blot analysis of apoE transgenic mice

Tail-derived DNA (5 µg) from newborn mice from the injected eggs was digested with BamHI and subjected to Southern blotting using the 1.7-kbp BamHI fragment of rat genomic apoE DNA as described under Methods. The probe hybridized to a 1.7-kbp band and a 1.2-kbp band in the samples from a rat as one copy control and a mouse of the same strain as negative control, respectively.

Based on Southern blot of rat tail-derived DNA as a one-copy positive control, it was estimated that the number of integrated copies of the rat apoE gene in the founder animals varied between 0.5 and 10. Out of eight transgenic founders, the offspring of four lines designated lines 4-16, 4-20, 4-26, 6-3, were consistently positive and were analyzed. To analyze mRNA levels of the integrated gene, total RNAs were prepared from the various organs of transgenic mice (F1). Prior to sacrifice for resection of organs, the animals were given water containing 20 mM $ZnSO_4$ to induce expression of the transgene under control of MTH promotor. Northern blot analysis was performed using two rat apoE specific oligonucleotide probes which did not hybridize with mouse apoE mRNA (Fig. 6).

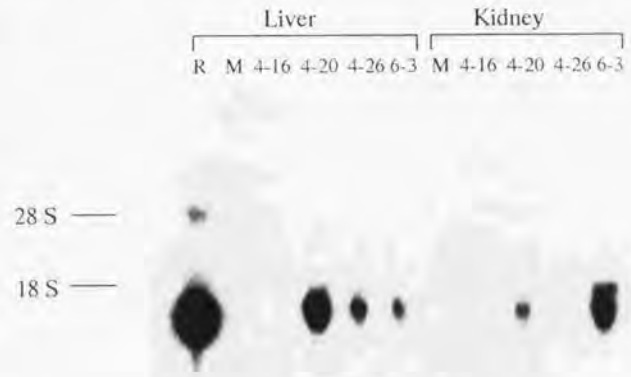


Figure 6. Northern blot analysis of apoE transgenic mouse tissue RNAs

Total RNA (5μg) from livers, kidneys from F1 transgenic mice pretreated with zinc ion were subjected to Northern blotting as described under Methods. The 5' end-labelled rat-specific oligonucleotide was used for the probe, which hybridized to rat apoE mRNA and not to mouse apoE mRNA. After longer exposure, expression of the integrated DNA was detectable in the brains from lines 4-26 and 6-3. Other tissues were also tested, however no significant expression was observed in any line.

Rat apoE mRNA in line 4-20 with three copies of the integrated gene was at high levels in the liver as compared with rat liver used as positive control. Lower levels were present in the livers from lines 4-26 with five copies. No detectable level was present in line 4-16, though it has the most integrated copies, 10. In spite of the very low level in the liver, line 6-3 with three copies has abundant rat apoE mRNA in the kidney. No significant levels of rat apoE mRNA were detected in other tissues from any line, only after longer exposure of the autoradiograms rat apoE mRNA was detected in the brains and spleens (data not shown). These results indicate that under control of MTHI promotor, high levels of apoE could be transcribed in the liver and/or in the kidney. Mouse metallothionein is expressed mainly in the liver, but also ubiquitously in the tissues. Previous reports on transgenic mice with MTHI promotor suggested that a considerable expression in the liver, kidney and intestine could be expected (17, 37). However, the selective and high expression in the kidney of our line 6-3 was unusual because pretreatment with zinc usually induces hepatic and not renal expression of MTH (38). Some unknown tissue factor(s) may exist for the renal expression of apoE. Even in the liver expression, a gene dosage effect was not observed in the transgenic lines with different numbers of integrated copies. These differences in expression among the transgenic lines or tissues with the same injected DNA would be caused by integration of the injected DNA at the different sites.

Heavy Metal Induction of Plasma ApoE in Transgenic Mice

To confirm the expression and secretion of the transgene, plasma was collected from the retroorbital plexus of F1 animals treated with zinc ion and examined by immunoblot analysis using a rat-specific polyclonal antibody. The rat apoE was detectable in all three lines of transgenic mice at relative molecular mass of 35 kDa corresponding to that of rat plasma used as positive control (Fig. 7a). The pooled plasma samples from six F1 animals of the high liver-expressing line 4-20 contained the largest amount of rat apoE. Those from low liver expressing line 4-26 had a lower plasma level. Besides, high kidney-expressing lines 6-3 also had a considerable level of plasma rat apoE.

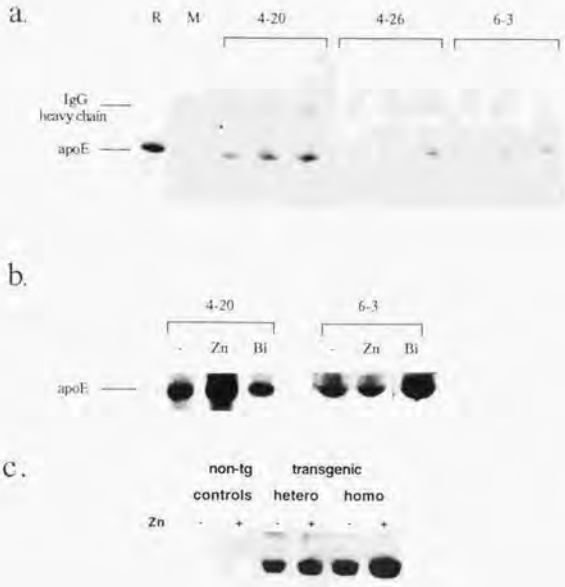


Figure 7. Immunoblot analysis of plasma from apoE transgenic mice

For treatment with zinc ion, the animals were given drinking water supplemented with 20 mM $ZnSO_4$ for one week. For treatment with bismuth ion, 1 μmol of Bi $(NO_3)_3$ (10 mM x 0.1 ml) was injected intracutaneously on the previous day. Plasma samples were drawn from the several F1littermates of each transgenic line (4-20, 4-26, 6-3) after a 12-hour fast. Portions (1 μl) of each sample or mixed plasma were subjected to immunoblot analysis using rat apoE-specific antibody as described in Methods. a. Plasma samples drawn from F1 littermates (n = 3) of each transgenic line after zinc treatment . M: mouse control plasma pooled from 20 animals of the same strain, R: rat control plasma. b. Heavy metal induction of rat apoE in mixed plasma samples from line 4-20 or 6-3 after treatment with zinc ion (Zn) or bismuth ion (Bi).

c. Plasma samples collected from female homozygotes (homo) of line 4-20 (n = 6), heterozygous (hetero) littermates (n = 16) and non-transgenic (non-tg) littermates as controls (n = 19) before (-) and after (+) the zinc treatment.

Smith et al.(15) suggested, because human apoE accumulates in the plasma of transgenic mice expressing in the kidneys, that an ectopic site of expression of an introduced gene may be permissive for the accumulation of its protein in plasma, and we confirmed their observation in the kidney-expressing line 6-3. The plasma rat apoE levels of lines 4-20 and 6-3 before and after administration of heavy metal ions were compared. As shown in Fig. 7-b, the amount of rat apoE in the plasma from the transgene of line 4-20 was 1.77-fold (n = 4) enhanced by induction with zinc ion and not by bismuth ion, while line 6-3 showed 2.48-fold increase after treatment with bismuth ion and no increase after zinc ion (n = 4). It was compatible with the previous report that the contents of methallothionein protein per tissue weight in the liver and the kidney of mouse were 2.6-fold and 4.9-fold increased after administration of zinc and bismuth compounds, respectively (38). Tissue-metal ion specificity may be related to the degree of accumulation of metal ions in the tissues (38).

To investigate the effect of overexpressed apoE on plasma lipoprotein metabolism, six female homozygotes for the transgene of line 4-20 were established as high expressors by mating the male and female animals of lines 4-20. Female heterozygous and non-transgenic littermates were used for comparison. Immunoblot analysis using rat apoE specific antibody (Fig. 7c) demonstrated that the level of rat apoE in homozygotes was twofold that in heterozygotes, suggesting expression from both integrated rat apoE genes. When the mice were given water supplemented with 20 mM zinc sulfate for one week, the plasma level of rat apoE was increased to 17.4 mg/dl by 1.6-fold (Table 1). The mouse apoE level of pooled non-transgenic mice (n=16) was estimated as 4.56 mg/dl from the analysis using polyclonal antibody against rat apoE which cross-reacts with mouse apoE.

Analysis of Plasma Lipoprotein Metabolism in Transgenic Mice

This overexpression of apoE caused marked alterations in plasma lipids (Table 1). Homozygotes exhibited a remarkable decrease of 43% in cholesterol level after the zinc administration as compared with zinc treated non-transgenic littermates (controls) ($p < 0.01$). The pretreatment level of this group was already 23% lower than untreated controls. In heterozygotes, the plasma cholesterol level after the zinc treatment was 20% lower ($p < 0.015$), although no significant difference was found before the treatment as compared with controls. Plasma triglycerides levels were also compared among the three groups. In homozygotes, remarkable decreases were observed both before and after the zinc treatment (by 64%, $p < 0.005$, and 68%, $p < 0.01$, respectively). Heterozygotes had a significantly lower pre-treatment level (66% of control pre-treatment level, $p < 0.05$), and further reduction was observed after the treatment (51% of control post-treatment level, $p < 0.001$). These data indicated that the overexpression of rat apoE in the transgenic mice, remarkably and dose-dependently, reduces both plasma cholesterol and triglycerides levels. ApoB level in homozygotes after zinc treatment was approximately 1/4 that in control mice.

To determine the changes in plasma lipoprotein profiles in the transgenic mice, gel filtration chromatography was performed. Elution patterns of cholesterol are shown in Fig. 8. In homozygotes after the zinc treatment, VLDL cholesterol was almost eliminated (3%) and an 85% decrease in LDL was observed as compared with zinc-treated controls. In addition, HDL cholesterol was decreased by 27%. Before the treatment, the VLDL, LDL and HDL cholesterol levels in homozygotes were already lower at 80%, 35%, 14%, respectively. In heterozygotes after zinc treatment, VLDL cholesterol and LDL cholesterol were 75% and 50% lower than zinc-treated controls, respectively. Triglycerides monitoring is shown in Fig. 9. In homozygotes, no obvious peaks were found, while controls showed a major peak in the VLDL fraction. The peak in the VLDL fraction in heterozygotes was decreased before the treatment and further reduced after the treatment.

Table 1. Effect of apoE expression on plasma cholesterol and triglyceride levels in transgenic mice.

	Zn treatment	rat apoE*	apoB*	cholesterol (n)	triglycerides (n)	cholesterol feeding cholesterol (n)
controls	before	-	25.0	91.9 ± 15.6 (16)	53.0 ± 18.3 (15)	- (5)
	after	-	29.7	95.0 ± 13.6	49.1 ± 21.2	194.4 ± 32.5
heterozygotes	before	5.9	21.1	93.0 ± 16.6 (19)	34.9 ± 9.7 (22)	- (5)
	after	9.3	15.9	76.8 ± 24.7	25.2 ± 9.3	115.7± 23.1
homozygotes	before	10.9	15.1	71.1 ± 12.4 (6)	19.2 ± 11.8 (5)	- (5)
	after	17.4	6.3	54.5 ± 19.4	16.0 ± 6.7	94.0 ± 9.1

mean ± SD mg / dl, * Values were determined from pooled samples of each group (mg / dl).

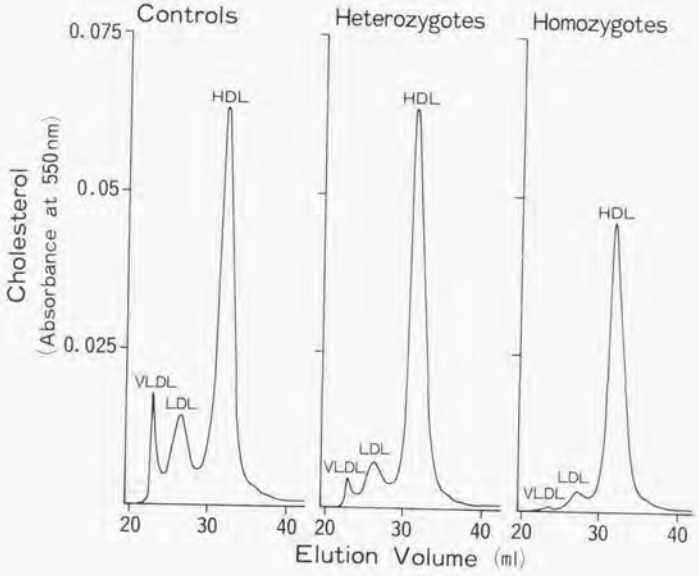


Fig.8 The profile of plasma lipoprotein cholesterol in transgenic mice by gel filtration chromatography.

A portion (10 µl) of pooled fasting plasma sample from each group after the zinc treatment was applied to high performance liquid chromatography system composed of TSK G3000SW (Tosoh Co, Tokyo) and Superose 6 HR (Pharmacia) as described in Materials and Methods. The peak positions of VLDL, LDL and HDL are indicated. The cholesterol levels (mg/dl) in VLDL and LDL calculated from the plasma total cholesterol and from the curve area in the elution pattern of HPLC were as follows. VLDL cholesterol: control 9.5 , heterozygotes 1.8, homozygotes 0.3, LDL cholesterol: control 17.2, heterozygotes 8.1, homozygotes 2.7.

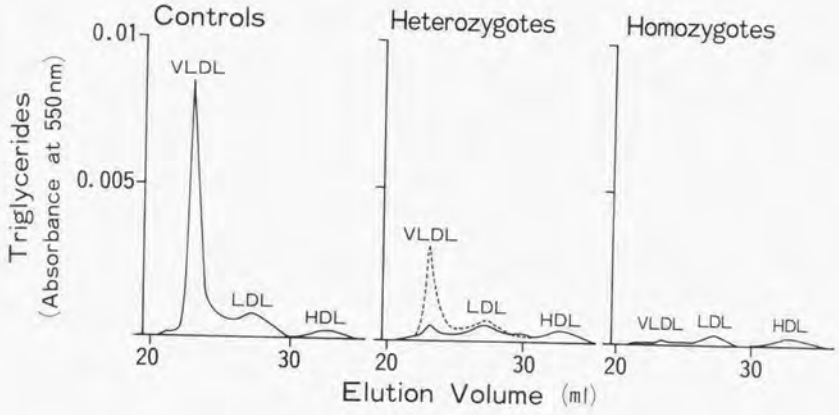


Fig.9. The profile of plasma lipoprotein triglycerides in transgenic mice by gel filtration chromatography.

The method is as described in Fig. 2. In the triglycerides monitoring in heterozygotes, the elution curves before (dotted line) and after (solid line) the zinc treatment are shown.

Immunoblot analysis of each lipoprotein fraction obtained by gel filtration chromatography was conducted to know the distribution of apoE among plasma lipoprotein and lipoprotein-unbound fractions. We used gel filtration chromatography to separate plasma lipoprotein classes because alternative ultracentrifugation method causes substantial loss of apoE from lipoproteins, especially VLDL, to lipoprotein-deficient plasma fraction during ultracentrifugation. As shown in Table 2, apoE distribution patterns of heterozygous transgenic mice and controls are similar. The data indicated that both endogenous mouse apoE and rat apoE from the transgene were distributed among all lipoprotein fractions and were associated almost entirely with lipoproteins. In homozygotes, apoE could not be detected in VLDL fraction indicating that disappearance of VLDL, and was distributed mainly on HDL and residually on LDL and in free form.

Table 2. Distribution of apoE among lipoprotein subclasses

	VLDL	LDL	HDL	not associated with lipoproteins
non-transgenic control	13.0	16.8	62.7	7.5
heterozygotes	15.5	18.5	60.9	5.0
homozygotes	0	8.6	83.0	8.9

% distribution of apoE is shown from immunoblot analysis of fractions of pooled plasma (n=5) after zinc treatment applied to gel filtration chromatography.

We examined effects of high cholesterol feeding on the plasma cholesterol level in the transgenic mice. High (1%) cholesterol feeding for ten days increased plasma cholesterol level of control animals by approximately twofold. In the transgenic mice, the rise in plasma cholesterol was much less, as shown in Table 1.

Time-coursed changes in plasma levels of retinyl palmitate after oral administration were compared among three groups. Retinyl palmitate secreted with intestinal lipoproteins, and presumably remains highly associated with the chylomicron-chylomicron remnant apoB48 particle until its removal from plasma. Because normal plasma level of retinyl palmitate is very low, it has been used as a marker for kinetics of chylomicrons and their remnants. The peak levels in heterozygotes and homozygotes (at 2 hour after administration) were 28% and 19% of that of controls, respectively (Fig. 10). This indicated increased clearance of chylomicrons and chylomicron remnants in transgenic mice.

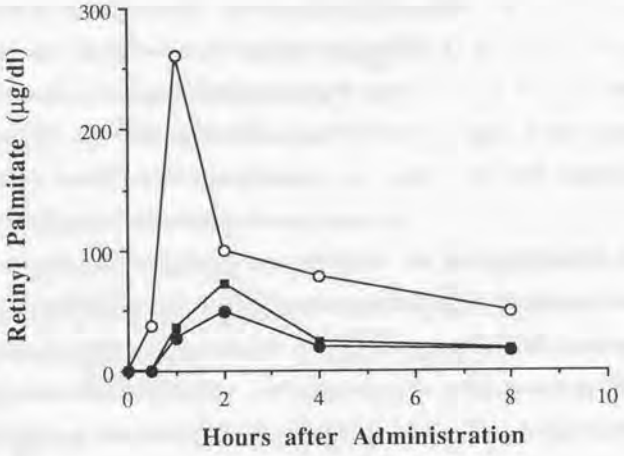


Fig. 10. Kinetics of Plasma Retinyl Palmitate after Oral Administration in transgenic mice.
Homozygotes (closed circles), heterozygotes (closed squares), and non-transgenic littermates (open circles) (n=3) were given retinyl palmitate (2mg) orally. Blood samples were drawn at indicated hours. Pooled plasma samples (100 μl) from each group were applied to HPLC to measure the levels of retinyl palmitate by fluorometry.

Discussion

Overexpression of apoE in transgenic mice caused dramatic changes in plasma lipids and lipoprotein profile. In the present study, we used metallothionein promotor in the injected DNA construct for the following reasons. First, metallothionein promotor has been commonly used in the production of transgenic mice overexpressing secretory proteins and cell surface receptors (37, 39). Second, the fusion gene of metallothionein promotor and apoE gene are presumed to overexpress apo E in the liver which is a physiologically primary organ producing apoE. And last, we can study the effect of increased plasma apoE by heavy metal administration under control of metallothionein promotor in the same animal. Previous reports on transgenic mice with a human apoE genomic gene demonstrated that multiple independent regulatory elements control the expression of the human apoE/CI gene locus in various tissues such as liver, kidney, skin and brain (14, 16). They reported that extended 3'flanking region including apoCI and CI pseudogene is required for the expression of apoE gene in the liver, whereas our DNA construct is free from this complex intrinsic regulation.

In the transgenic mice that we have established, the overexpressed apoE was entirely associated with all plasma lipoproteins and an unassociated free form of apoE was minimal (Table 2), as reported in the transgenic mice expressing human apoE in the kidney (15). Since rat and mouse apoE amino acid sequences have a high homology (18 , 32), the activity of overexpressed apoE in plasma lipoprotein metabolism would be similar to that of mouse apoE. The effects of overexpressed apoE on plasma lipoprotein lipid levels were dose-dependent as shown in Table 3. There were four stages of the plasma apoE levels in mice; the level of controls, zinc-untreated heterozygotes (6 mg/dl, low expression), treated heterozygotes which is similar to that of untreated homozygotes (10 mg/dl, middle expression), and treated homozygotes (17 mg/dl, high expression).

Table 3 Dose-related effect of apoE on plasma lipoprotein classes

Zn treatment	heterozygotes before	heterozygotes after	homozygotes before	homozygotes after
rat apoE (mg/dl)	5.9	9.3	10.9	17.4
plasma cholesterol	→	↓	↓	↓↓
VLDL cholesterol	↓↓	↓↓↓	↓↓↓	~0
LDL cholesterol	↓	↓↓	↓↓	↓↓↓
HDL cholesterol	↑	→	↓	↓↓
plasma triglycerides	↓	↓↓	↓↓↓	↓↓↓
VLDL triglycerides	↓↓	↓↓↓	~0	~0

Changes as compared with corresponding control levels are shown. →; less than 10% decrease,
↓; 10-25% decrease, ↓↓; 25-60% decrease, ↓↓↓; 60-95% decrease, ~0; more than 95% decrease

A decrease in plasma cholesterol level was observed at the plasma apoE level of middle expression. It is consistent with the data reported by Smith et al. that no significant changes in plasma cholesterol levels were found in the transgenic mice with the level of 3-4 mg/dl of human apoE corresponding to our low expression level (15). Decreases in plasma VLDL- and LDL-cholesterol levels were already observed at the plasma apoE level of low expression, and the greater plasma apoE level, the more prominent decreases in plasma VLDL- and LDL-cholesterol levels were observed. In contrast, a decrease in plasma HDL-cholesterol level required the plasma apoE level of high expression. In the same plasma apoE level, decreases in plasma triglycerides were much more remarkable than those in plasma cholesterol. Plasma VLDL triglycerides were remarkably reduced even at the plasma apoE level of low expression and were eliminated at both levels of middle and high expression. Supporting the significant reduction in plasma lipoprotein

lipids in transgenic mice, marked reduction in plasma apoB levels of treated homozygotes was observed, which indicates the elimination of most of lipoproteins containing apoB.

Together with enhanced clearance of chylomicrons as shown in Fig. 10, the data suggested that apoE has the most profound effect on the metabolism of triglycerides rich lipoproteins such as chylomicron and VLDL, and the least remarkable in HDL. VLDL particles, which are synthesized in the liver, can be associated with overexpressed apoE in the liver, achieve a high affinity for LDL receptor, resulting in a rapid plasma clearance of VLDL through receptor-mediated pathways, as we have demonstrated an increased uptake of VLDL in CHO-MAEII as an *in vitro* model (Fig. 3). The reduction in LDL cholesterol could be caused by a reduced production in a metabolic cascade from VLDL of which clearance was enhanced. Possibly, additional apoE might also enhance the clearance of LDL particles, although *in vitro* studies suggested no effect of exogenous apoE on the cellular uptake of human LDL (10-12). Since some HDL particles with apoE can be taken up by LDL receptor (39), plasma clearance of HDL might also be enhanced to some extent due to multiple molecules of apoE on HDL in homozygotes.

The molecular structure of rat apoE is contributory to the marked decrease in lipoproteins containing apoB100 in this transgenic line. Human apoE has three major isoforms, apoE2, E3 and E4 according to amino acid polymorphism (1, 40). These isoforms have different preferences in distribution among plasma lipoprotein classes. ApoE4 tends to be associated with VLDL more preferentially and with HDL less as compared with apoE3, the wild type. It has been reported that plasma lipoproteins containing apoE4 are more receptor-active than those containing apoE3 because a substantial proportion of apoE3, and not apoE4, forms a homodimer or heterodimer with apoAII in plasma, which are defective in receptor binding (41). Furthermore, kinetic studies after injection of each isoform have revealed that the clearance of apoE4 is more rapid than that of apoE3 partly due to differential distribution on lipoprotein classes (42). Rat apoE as well as mouse apoE are the human apoE4 type (24, 32), therefore, the transgene product would be most active among apoE phenotypes in determining the clearance of lipoproteins.

It was reported that overexpression of LDL receptor in transgenic mice eliminates plasma LDL cholesterol (39) and prevented diet-induced hypercholesterolemia (43). These observations indicated the importance of the LDL receptor in catabolism of lipoproteins containing apoB. In the present study, we studied the effect of overexpressed apoE on diet-induced hypercholesterolemia from the aspect of increased ligand affinity for its receptor, and the similar function of apoE in the prevention of diet-induced hypercholesterolemia was demonstrated. There are two possible mechanism for the prevention of development of hypercholesterolemia in apoE transgenic mice fed a high cholesterol diet. One is accelerated clearance of cholesterol-rich lipoproteins through LDL receptor because apoE-rich lipoproteins have high affinity to LDL receptor (1), and another is alternative pathways such as chylomicron remnant receptors (that might be LDL receptor-related protein) which are considered to be specific to apoE-rich lipoproteins (44-47) and are not suppressed by cholesterol load (48, 49), although these pathways have not been fully understood. In general, high cholesterol feeding causes suppression of hepatic LDL receptor activities resulting in hypercholesterolemia because dietary cholesterol induces increased cholesterol content in the liver (43, 50). Therefore, lipoproteins containing several molecules of apoE might be taken up by the liver through pathways other than LDL receptor in transgenic mice overexpressing apoE during cholesterol feeding. The resistance against hypercholesterolemia in our transgenic mice suggests possible involvement of the alternative pathways specific to apoE in the removal of apoE rich lipoproteins from the blood. Enhanced clearance of chylomicron remnants in the transgenic lines might be also due to an increased affinity for this putative receptor.

In the present study, we demonstrated that apoE is an essential apolipoprotein which determines the level and profile of lipoproteins, especially lipoproteins containing apoB, by influencing the interaction between lipoprotein receptors and lipoproteins. These transgenic lines will also provide a good model to facilitate evaluation of multi-functions of apo E in numerous tissues. In particular, the function of lipoproteins containing excess apoE in atherogenic process will be clarified in this animal model.

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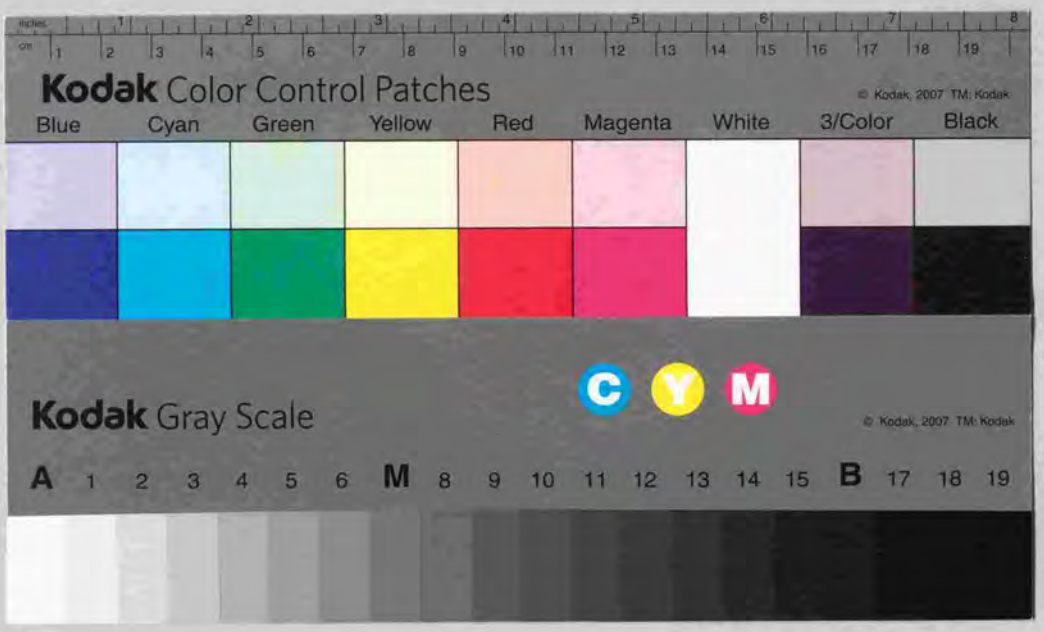
Lipoprotein Metabolism in Transgenic Mice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