Apolipoprotein E-deficient mice created by systemic administration of antisense oligodeoxynucleotides: a new model for lipoprotein metabolism studies

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Abstract

Atherosclerotic cardiovascular disease results from complex interactions among multiple genetic and environmental factors. Thus, it is important to elucidate the influence of each factor on cholesterol metabolism. For this purpose, transgenic/gene-targeting technology is a powerful tool for studying gene functions. However, this technology has several disadvantages such as being time consuming and expensive. Accordingly, we established new animal models using *in vivo* gene transfer technology. In this study, we examined the feasibility of the creation of a new animal model for the study of atherosclerosis. We hypothesized that apolipoprotein (apo) E-deficient mice can be created by systemic administration of antisense apo E oligodeoxynucleotides (ODN) coupled to the HVJ-liposome complex. Initially, we examined the localization and cellular fate of FITC-labeled antisense ODN administered intravenously. FITC-labeled ODN transfection by the HVJ-liposome method resulted in fluorescence in the liver, spleen and kidney, but not in other organs such as brain. Moreover, fluorescence with the HVJ-liposome method was sustained for up to 2 weeks after transfection, which resulted in a striking difference from transfection of ODN alone or ODN in liposomes without HVJ, which showed rapid disappearance of fluorescence (within 1 day). Given these unique characteristics of the HVJ-liposome method, we next examined transfection of antisense apo E

ODN by intravenous administration. Transfection of antisense apo E ODN resulted in a marked reduction of apo E mRNA levels in the liver, but no change in apo B and β -actin mRNA levels. In mice fed a normal diet, a transient increase in cholesterol and triglyceride levels was observed in the antisense apo E-treated group, but they returned to normal levels by 6 days after transfection. Similar findings were also found in mice fed a high cholesterol diet. Neither scrambled nor mismatched ODN resulted in any increase in cholesterol. To make chronic hypercholesterolemic mice, we therefore performed repeated injections of apo E antisense ODN. Whenever antisense apo E ODN were injected, mice showed a transient increase in cholesterol and triglyceride. Cumulative administration of antisense apo E ODN resulted in a sustained increase in cholesterol for up to 3 weeks after the last transfection. Finally, mice treated with repeated injections of antisense apo E every week developed sustained hypercholesterolemia and hypertriglyceridemia until withdrawal of injections. Apolipoprotein-deficient mice created by intravenous administration of antisense ODN are a promising new animal model to help understand the role of apolipoprotein *in vivo* and develop a new drug therapy targeting apolipoprotein.

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Introduction

Atherosclerotic cardiovascular disease results from complex interactions among multiple genetic and environmental factors. Among these factors, apolipoprotein (apo) E has been of interest because of its known genetic variant (Zannis & Breslow 1981, Utermann *et al.* 1982). Apo E is one of the apolipoproteins that coat the surface and

mediate the metabolism of lipoprotein particles. It is synthesized primarily in the liver, although synthesis has been documented in the brain and other tissues. One of its most important functions is to serve as a high affinity ligand for the apo B and low density lipoprotein (LDL) receptors and for the chylomicron-remnant receptor, thereby allowing the specific uptake of apo E-containing particles by the liver. In addition to lipoprotein transport,

apo E has been postulated to play a role in reverse cholesterol transport, local redistribution of cholesterol within tissues, immunoregulation, cell proliferation, and differentiation (Mahley 1988). Uniquely, a frequent genetic variant of human apo E, apo E2, differs from the most common form, apo E3 (Zannis *et al.* 1982). The amino acid substitution in the LDL receptor binding region reduces the binding activity of apo E2 to less than 2% relative to that of apo E3 (Weintraub *et al.* 1987). As a clinically important point, homozygosity for the gene apo E2 is associated with type III hyperlipoproteinemia accompanied by high cholesterol and triglyceride levels, resulting in the development of atherosclerosis (Mahley & Rall 1989). Recent progress in molecular biology, such as transgenic technology, has enabled the study of specific gene function, which classical pharmacological experiments could not dissect out. Over-expression of the apo E gene in mice resulted in a decrease in cholesterol and prevention of atherosclerosis (Shimano *et al.* 1992). Recently, there has been increased interest in the role of apo E in cholesterol metabolism, because mice lacking the apo E gene have been created by gene targeting technology. These mice show severely high cholesterol and relatively high triglyceride levels in response to a Western-type diet (Plump *et al.* 1992, Zhang *et al.* 1992). Although transgenic/gene targeting technology is a powerful tool for studying gene functions, this technology has several disadvantages: (1) it is time consuming and expensive, (2) it is dependent on developmental and systemic effects, and (3) it is associated with difficulty in the experimental protocol. Accordingly, we have established a new animal model using *in vivo* gene transfer technology (Dzau *et al.* 1994). For example, *in vivo* transfer of the renin gene directly into adult rat liver resulted in hypertension (Tomita *et al.* 1993), and *in vivo* transfer of the angiotensin converting enzyme gene into blood vessels resulted in vascular hypertrophy independent of a systemic effect (Morishita *et al.* 1994a). In this study, we applied antisense technology to study specific gene functions, besides gene targeting technology. Here, inhibition of apolipoprotein E by the systemic administration of antisense oligodeoxynucleotides (ODN) using the HVJ (Hemagglutinating Virus of Japan)-liposome method resulted in hypercholesterolemia and hypertriglyceridemia as a new animal model ('antigenic' model) of lipoprotein metabolism.

Materials and Methods

Synthesis of oligodeoxynucleotides and selection of target sequences

The sequences of ODN against mouse apo E used in this study were as follows: antisense: 5'-CCA-CAG-AGC-CTT-CAT-CTT-C-3', sense: 5'-GAA-GAT-GAA-GGC-TTC-GTG-G-3' (-4 to +15 of the mouse sequence; these sequences are identical in mouse and

human apolipoprotein E). We also synthesized scrambled and a 4-base pair mismatched ODN of the apolipoprotein E antisense ODN (scrambled ODN: 5'-CTT-CGT-CGG-TAC-CGT-CTT-C-3', mismatched ODN: 5'-CCA-CAG-TGC-CCT-CGT-TTT-C-3'). Synthetic ODN were purified over a NAP 10 column (Pharmacia), and quantitated by spectrophotometry (Morishita *et al.* 1993, 1994b).

Preparation of HVJ-liposome

Phosphatidylserine, phosphatidylcholine, and cholesterol were mixed in a weight ratio of 1:4:8:2 (Tomita *et al.* 1993, Morishita *et al.* 1993, 1994a,b). Dried lipid was hydrated in 200 µl balanced salt solution (BSS; 137 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl, pH 7.6) containing ODN. The control group as a negative control did not contain ODN (BSS 200 µl). Liposomes were prepared by shaking and sonication. Purified HVJ (Z strain) was inactivated by UV irradiation (110 erg/mm²/s) for 3 min just before use. The liposome suspension (0.5 ml, containing 10 mg lipid) was mixed with HVJ (10 000 hemagglutinating units) in a total volume of 4 ml BSS. The mixture was incubated at 4 °C for 5 min and then for 30 min with gentle shaking at 37 °C. Free HVJ was removed from the HVJ-liposome by sucrose density gradient centrifugation.

In vivo transfection of FITC-labeled antisense ODN

FITC-labeled phosphorothioate antisense ODN were provided by Clontech Inc. (Palo Alto, CA, USA). FITC was used to label the 3' and 5' ends of the antisense apo E ODN using fluorescein-ODN phosphoramidite (Morishita *et al.* 1994b). Transfer of phosphorothioate ODN was performed according to three protocols: (1) 200 µl HVJ complex with FITC-labeled phosphorothioate ODN (3 µM) was used; (2) 200 µl ODN in liposomes without HVJ particles: liposome complex with ODN (3 µM), but without HVJ particles was used; (3) 200 µl FITC-labeled ODN (30 µM) was used. Each complex was injected into mice via the tail vein. At 30 min, 12 and 24 h, and at 3, 7 and 14 days after transfection, mice were killed, and tissues were removed after perfusion-fixation with 4% paraformaldehyde under physiological pressure (110 mmHg). Sections were examined by fluorescent microscopy, after staining with erichrome black T solution; elastic fibers stained dark red and were readily distinguishable from the specific FITC-labeled antisense ODN (data not shown) as previously described (Kittelberger *et al.* 1989, Morishita *et al.* 1994b). We also performed quantitative measurement of fluorescence to confirm the efficiency of FITC-labeled ODN transfection with the HVJ-liposome method (1 µM). FITC-labeled ODN with the HVJ-liposome complex was intravenously injected into the tail vein. For measurement of

Figure 1 (a) Representative fluorescence microscopy of various organs transfected with FITC-labeled ODN by the HVJ-liposome method at 1 day after transfection. (b–d) Comparison of passive uptake and the HVJ-liposome method in liver (b), spleen (c) and kidney (d). HVJ, mice transfected with FITC-labeled ODN by the HVJ-liposome method; ODN, mice transfected with FITC-labeled ODN without the HVJ-liposome method; 30M, at 30 min after transfection; DAY 1, at 1 day after transfection; DAY 4, at 4 days after transfection.

fluorescence, tissues were then extracted at 1 day after transfection. They were homogenized in 1 ml PBS, centrifuged and 400 μ l of the supernatant were used for measurement with a fluorescence meter (RF1500 spectrofluorophotometer, Shimadzu) (excitation, 493 nm; emission, 520 nm).

Reverse transcription (RT)-PCR and Northern blot

First, 200 μ l HVJ-liposome complex containing sense or antisense ODN (15 μ M in liposomes/body) were injected into the tail vein of mice fed a normal or a high cholesterol diet. One day after transfection, the liver was infused with sterile PBS, then frozen immediately in liquid nitrogen

and stored at -80°C until RNA extraction. RNA was extracted using RNAzol (Tel-Test Inc., Dallas, Texas, USA). Extracted RNA was quantified by measuring absorbance at 260 nm. PCR amplification of 0.5 μg aliquots of extracted RNA was performed with oligonucleotide primers complementary to the apo E gene. The 5' primer was 5'-GAG-CTG-GAG-GAA-CAG-CTG-GGT-3'; the 3' primer was 5'-CTC-GAC-CTC-CTT-GTC-GAC-CCA-3' (Wang *et al.* 1989). Primers complementary to the apo B gene were also used (the 5' primer was 5'-GGA-ATT-CAT-TAT-CTG-AAT-GCA-TC-3'; the 3' primer was 5'-GGG-ATC-CAT-GAT-TCG-ATC-AAT-AA-3') (Xiong *et al.* 1992). Extreme care was taken to avoid contamination of tissue samples with trace amounts of experimental RNA. Aliquots of RNA derived from tissues were amplified simultaneously by PCR and compared with a negative control (primers without RNA). The amplification protocol was as follows: 95°C for 30 s, 55°C for 30 s, 72°C for 1 min. Amplification products were electrophoresed through 2% agarose gels stained with ethidium bromide. Thirty and forty cycles of PCR amplification were used (Morishita *et al.* 1993, 1994b). As a control for the integrity of the extracted RNA as well as for the PCR protocol, an aliquot of each RNA sample was also amplified with oligonucleotide primers complementary to the mouse β -actin gene (donated by Clontech). Extreme care was taken to avoid contamination of tissue samples with trace amounts of experimental RNA. Aliquots of RNA from both experimental and negative control tissues were amplified simultaneously, with the same reagents, by individuals who were blinded to the identity of the samples. To check if the detected band was due to the transcription of the gene, RNA samples treated with RNase A were amplified simultaneously as a negative control. These samples did not result in a visual band (data not shown). At least three aliquots of each RNA sample were subjected to separate PCR amplifications in all experiments.

We also examined apo E mRNA expression using Northern blot analysis in a standard manner (Morishita *et al.* 1992). The 5' PCR primer of apo E was labeled by an end-labeling kit (donated by Clontech), and used as a probe.

Serum cholesterol and triglyceride levels

Male C57/BL6 mice (12 weeks; Charles River Laboratories, Boston, MA, USA) were used for experiments. The mice were maintained and bred under conventional conditions with food and tap water available *ad libitum*, except on the days of blood sampling, and a 12 h light–12 h darkness photoperiod. Cholesterol, triglyceride and high density lipoprotein (HDL) assays were performed as recommended by the manufacturer of the assay kit (Sigma Immunochemicals, St Louis, MO, USA).

Transfection of antisense ODN

Mice transfected with sense, scrambled, mismatched or antisense ODN were examined for measurement of cholesterol and triglyceride levels. The following protocols were performed. (1) Systemic administration of ODN via the tail vein was performed on day 0, and blood was obtained on days 1, 2, 3 and 6 after transfection. (2) ODN transfection was performed on days 0, 1, and 2 to study the cumulative effects of ODN. (3) ODN transfection was performed on days 0, 7, and 16 to study the effect of repeated injections. (4) ODN transfection was performed once or twice a week to follow the changes in serum cholesterol and triglyceride levels. After transfection, blood specimens were collected periorbitally into heparin-containing microtubes to study the time-course of changes in glucose level. Mice were fasted for at least 8 h before blood sampling. A normal diet (without cholesterol) or a high cholesterol diet (1% cholesterol, 0.2% cholic acid and 20% coconut oil; Dyets Inc., Bethlehem, PA, USA) was administered.

Statistical analysis

All values are expressed as means $\pm$ S.E.M. Analysis of variance with Duncan's test was used to determine the significance of differences in multiple comparisons. $P < 0.05$ was considered to be statistically significant.

Results

Detection of FITC-labeled antisense ODN after systemic transfection

Initially, we studied the localization of FITC-labeled antisense ODN to evaluate the character of various ODN delivery transfer methods. At 30 min after transfection, fluorescence was detected in the liver, spleen and kidney (tubular cells, but not mesangial cells) with the HVJ-liposome method, whereas no fluorescence was observed in brain, testis, lung (Fig. 1a), heart, muscle and intestine (data not shown). In contrast, passive uptake of FITC-labeled antisense ODN resulted mainly in the kidney, with little in the liver and spleen (Fig. 1b–d). In other tissues, no fluorescence was observed with passive uptake. Interestingly, a striking difference in the time course of fluorescence was observed between the HVJ-liposome method and passive uptake (Fig. 1b–d). Rapid disappearance of fluorescence (within 1 day) was found with passive uptake, whereas sustained fluorescence was observed with the HVJ-liposome method up to 2 weeks after transfection. Finally, we studied the role of HVJ particles in systemic administration of ODN. Transfection of FITC-labeled antisense ODN in the liposome complex without HVJ particles resulted in the same localization as with the HVJ-liposome method (data not shown).

Figure 2 (a) Detection of apo E, B and β -actin gene expression in liver of sense and antisense apo E ODN-transfected mice by RT-PCR. (b) Semiquantitative RT-PCR analysis of apo E mRNA in liver of sense (S) and antisense (AS) apo E ODN-transfected mice. (c) Northern blot of apo E mRNA in liver of sense and antisense apo E ODN-transfected mice. sense, RNA from the liver of mice transfected with sense apo E ODN; antisense, RNA from the liver of mice transfected with antisense apo E ODN; Apo E, apo E mRNA; Apo B, apo B mRNA; ACTIN, β -actin mRNA.

However, rapid disappearance of fluorescence was observed with the liposome-mediated method without HVJ, the same as for passive uptake. We confirmed the prolongation of ODN half-life with the HVJ-liposome method, consistent with previous reports (Morishita *et al.* 1994c, 1996). Untreated or HVJ complex without FITC-labeled antisense ODN resulted in no specific fluorescence (data not shown). Fluorescence resulting from FITC-labeled antisense ODN was easily distinguished from background auto-fluorescence. Systemic infusion of free FITC did not result in specific fluorescence in the tissues (data not shown), demonstrating that this fluorescence was specific for FITC-labeled ODN. Marked accumulation of ODN in the liver using the HVJ-liposome method was also confirmed by the measurement of fluorescence. Transfection of FITC-labeled ODN by the HVJ-liposome method resulted in a significant ($P < 0.01$ vs other tissues) increase in fluorescence detected in the liver as compared with other tissues at 1 day after transfection (liver:

46 500 $\pm$ 5600, spleen: 3600 $\pm$ 500, kidney: 2300 $\pm$ 400 fluorescence intensity, heart: not detected).

Specificity of antisense apo E ODN

To study the effect of systemic administration of antisense apo E ODN, initially we performed RT-PCR of liver. RT-PCR using RNA of mice transfected with antisense apo E ODN showed no visual band at 35 cycles, whereas RT-PCR from RNA of mice transfected with sense ODN yielded a clear visual band (Fig. 2a). Semiquantitative RT-PCR also showed a consistent decrease in apo E mRNA level in antisense ODN-treated mice (Fig. 2b). Moreover, the specificity of the antisense ODN effect was supported by the observation that no significant change in apo B and β -actin mRNA levels was observed in mice transfected with antisense apo E ODN (Fig. 2a). These findings were also supported by Northern blot analysis.

Table 1 Effect of mismatched and scrambled ODN on serum cholesterol and triglyceride levels in normal mice. Values=transfected mice – untransfected mice with vehicle injection and are expressed as means ± S.E.M.

	Antisense	Mismatched	Scrambled	Significance
Day				
Cholesterol				
1	20.0 ± 9.7*	– 1.7 ± 7.2	– 3.2 ± 9.1	*P<0.01
2	9.5 ± 4.6*	0.6 ± 2.9	– 2.7 ± 4.7	*P<0.01
3	20.6 ± 10.3*	2.1 ± 9.4	– 2.1 ± 9.5	*P<0.01
6	6.1 ± 3.6	3.1 ± 8.1	– 0.4 ± 8.4	NS
Triglyceride				
1	18.0 ± 6.2*	4.3 ± 3.9	3.7 ± 2.9	*P<0.01
2	21.7 ± 8.4*	– 2.6 ± 2.0	1.0 ± 11.1	*P<0.01
3	25.8 ± 7.1*	7.4 ± 7.3	– 9.4 ± 5.2	*P<0.01
6	4.3 ± 6.6	– 5.7 ± 4.0	– 10.0 ± 8.2	NS

NS=not significant.

Transfection of antisense apo E ODN decreased hepatic apo E mRNA levels as compared with sense ODN (Fig. 2c).

Effect of systemic administration of antisense apo E ODN in mice on a normal diet

Given that the existence of FITC-labeled antisense ODN was detected in the liver, spleen and kidney up to 2 weeks after transfection, we hypothesized that local inhibition of apo E in the liver by systemic transfection of antisense apo E ODN via the tail vein would modulate cholesterol metabolism. Accordingly, we studied the effect of a single systemic administration of antisense apo E ODN in mice on a normal diet. We measured serum cholesterol and triglyceride levels in mice transfected with sense and antisense apo E ODN. Cholesterol concentration was increased from day 1 to day 3 in antisense ODN-transfected mice after transfection, but on day 6 after transfection there was no significant difference between sense and antisense ODN-transfected mice (Fig. 3a,b). Similarly, serum triglyceride concentration was increased in antisense ODN-transfected mice as compared with sense ODN-transfected mice (Fig. 3c), while there was no significant change in HDL-cholesterol (data not shown). The specificity of antisense ODN was also supported by the observation that systemic administration of mismatched or scrambled ODN did not result in any significant change in cholesterol and triglyceride levels (Table 1).

Effect of systemic administration of antisense apo E ODN in mice on a high cholesterol diet

To investigate the feasibility of the antisense strategy to establish a new knockout model, we studied the effect of intravenous transfection of antisense or sense ODN in mice on a high cholesterol diet, given the susceptibility of

C57/B6 mice to the development of atherosclerosis in response to a high cholesterol diet. A single systemic administration of antisense ODN increased serum cholesterol and triglyceride levels as compared with sense ODN, similar to mice on a normal diet (Fig. 4a–c).

Given the transient increase in cholesterol in antisense ODN-transfected mice, it is necessary to achieve long-term inhibition of apo E in order to study lipoprotein metabolism. Therefore, we hypothesize that repeated injection of antisense ODN would prolong the increase in cholesterol levels. Indeed, an increase in cholesterol levels was observed in antisense ODN-transfected mice for up to 24 days after the last transfection by cumulative injection (days 0, 1 and 2) (Fig. 5). Alternatively, we examined whether repeated injection of antisense ODN would achieve the same effect on the increase in cholesterol levels. As shown in Fig. 6a and b, repeated injection of antisense apo E ODN on day 7 and day 16 after the first injection resulted in a sustained increase in cholesterol and triglyceride levels for up to 23 days. Finally, we tried to maintain a high serum cholesterol concentration by systemic administration of antisense apo E ODN. Our present study showed the successful establishment of high cholesterol mice by local targeting of hepatic apo E with antisense ODN, as shown in Fig. 7a and b. After stopping injection of antisense apo E ODN, the high cholesterol levels gradually returned to the same levels as those in sense ODN-transfected mice.

Discussion

Atherosclerosis is a major cardiovascular risk factor for myocardial infarction and cerebrovascular disease. Although the pathophysiology of atherosclerosis is quite complicated, it is unquestionable that abnormality of lipid metabolism such as a high cholesterol level causes lipid

Figure 3 (a) Effect of antisense apo E ODN transfection on plasma cholesterol and triglyceride levels in mice on a normal diet at 3 days after transfection. S, mice transfected with sense ODN; AS, mice transfected with antisense ODN. ** $P < 0.01$ vs S. (b and c) Time-course of changes in levels of (b) cholesterol and (c) triglyceride in antisense apo E ODN-transfected mice (normal diet). * $P < 0.05$, ** $P < 0.01$ vs mice transfected with sense ODN. Each group contained 6 animals. Values are antisense ODN – sense ODN.

Figure 4 (a) Effect of antisense apo E ODN transfection on plasma cholesterol and triglyceride levels in mice on a high cholesterol diet at 3 days after transfection. S, mice transfected with sense ODN; AS, mice transfected with antisense ODN. ** $P < 0.01$ vs S. (b and c) Time-course of changes in levels of (b) cholesterol and (c) triglyceride in mice transfected with antisense apo E ODN (high cholesterol diet). * $P < 0.05$, ** $P < 0.01$ vs mice transfected with sense ODN. Each group contained 6 animals. Values are antisense ODN – sense ODN.

deposition in the vascular wall. Especially, apolipoproteins such as apo E and apo (a) have an important role in the development of atherosclerosis (Lawn *et al.* 1992, Plump

et al. 1992, Zhang *et al.* 1992). It is well known that the plasma apo (a) level, which is determined by genotype, is an independent risk factor for myocardial infarction (Wade

Figure 5 Effect of cumulative injection of antisense apo E ODN on cholesterol level (high cholesterol diet). * $P<0.05$, ** $P<0.01$ vs mice transfected with sense ODN. Each group contained 6 animals. Values are antisense ODN – sense ODN. Arrows show transfection.

et al. 1993). Apo E is also at the center of interest, because patients with apo E deficiency have a lipoprotein pattern resembling that seen in type III hyperlipoproteinemia and also develop atherosclerosis and xanthomas (Ghiselli *et al.* 1981, Schaeffer *et al.* 1986, Mabuchi *et al.* 1989, Mahley & Rall 1989). Moreover, a common genetic variation of apo E in human populations is also known (Zannis *et al.* 1982).

Recent progress in molecular biology has enabled the study of the specific functions of lipoprotein genes. Using transgenic technology, overexpression or tissue-specific expression of lipoprotein genes can be analyzed. Alternatively, the complete inhibition of a gene can also be performed using gene targeting technology. Indeed, these technologies are quite powerful and useful for the study of lipoprotein metabolism. For example, overexpression of apo E prevented the development of atherosclerosis (Shimano *et al.* 1992). In contrast, mice lacking apo E showed spontaneous development of hypercholesterolemia and arterial lesions (Plump *et al.* 1992, Zhang *et al.* 1992). However, the transgenic/gene targeting technology has several disadvantages: (1) it is time consuming and costly, (2) the effect of the over-expressed transgene is exerted throughout development, and (3) it is difficult to exclude the potential contribution of the systemic effect of transgene expression. If the fate of knockout mice lacking the targeted gene is lethal, it is difficult to test the specific functions by transgenic or gene targeting techniques. In those cases, the gene transfer approach may be an alternative method.

Apo E is synthesized primarily in the liver, although synthesis has been documented in virtually every tissue. Therefore, hypercholesterolemia in apo E-deficient mice

Figure 6 Effect of repeated injection of antisense apo E ODN on cholesterol (a) and triglyceride (b) levels (high cholesterol diet). * $P<0.05$, ** $P<0.01$ vs mice transfected with sense ODN. Each group contains 6 animals. Values are antisense ODN – sense ODN. Arrows show transfection.

Figure 7 Effect of multiple repeated injection of antisense apo E ODN on cholesterol (a) and triglyceride (b) levels (high cholesterol diet). * $P<0.05$, ** $P<0.01$ vs mice transfected with sense ODN. Each group contained 6 animals. Values are antisense ODN – sense ODN. Arrows show transfection.

created by gene targeting may not reflect the deficiency of hepatic apo E. The specific inhibition of hepatic apo E would be useful to study the role of apo E. To create hepatic apo E-deficient mice, we examined the systemic administration of antisense apo E ODN. Towards this aim, ODN were administered intravenously via the tail vein. However, passive uptake of ODN resulted in little accumulation in the liver and spleen, and also a short half life (less than 1 day). Next, we transfected ODN in liposomes (without HVJ), given the utility of liposome-mediated delivery. Indeed, transfection of ODN in liposomes resulted in strong accumulation in the liver and spleen, but the short half-life could not be avoided (fluorescence disappeared within 1 day). Finally, we employed the HVJ-liposome method as the delivery system of ODN. As shown in this study, ODN in the HVJ-liposome complex could be transfected selectively into the liver, spleen and renal tubular cells. The localization of ODN in the HVJ-liposome complex was very different from that with passive uptake. Moreover, the

HVJ-liposome method resulted in marked prolongation of the half-life of ODN with persistence for at least 2 weeks after transfection. Given the unique character of the HVJ-liposome method, we transfected antisense apo E ODN in the HVJ-liposome complex by systemic injection.

Systemic administration of antisense apo E ODN resulted in selective inhibition of apo E mRNA expression in the liver, while there was no change in mRNA levels of apo B and β -actin. In apo E-deficient mice created with antisense ODN, a transient increase in cholesterol and triglyceride levels could be observed as compared with sense ODN-treated mice. The same phenomenon was also observed in mice fed a high cholesterol diet. The specificity of antisense ODN was also supported by the observation that neither scrambled nor mismatched ODN resulted in an increase in cholesterol and triglyceride. However, the magnitude and duration of the increase in cholesterol did not seem sufficient to study the specific role of apolipoprotein, especially compared with transgenic or knock-out mice. To address this issue, the cumulative effects of transfection of antisense ODN were examined. The cumulative transfection of antisense apo E ODN resulted in a relatively long-term increase in cholesterol (up to day 24). Alternatively, repeated transfection of antisense apo E ODN was also performed. An increase in cholesterol and triglyceride was observed in response to every injection of apo E ODN.

Finally, we tried to maintain a sustained increase in cholesterol and triglyceride. Repeated systemic injections of antisense apo E ODN created apo E-deficient mice who showed a sustained increase in cholesterol and triglyceride over 3 months. The magnitude of the increase in cholesterol (49 mg/dl; from 137 to 186 mg/dl) was relatively small as compared with that in homozygous gene targeted mice (cholesterol; from 132 to 1821 mg/dl), but more severe than that in heterozygotes (9 mg/dl; from 132 to 141 mg/dl). Reflecting the small increase in cholesterol level, the abdominal aorta and brachiocephalic artery showed no evidence of atherosclerotic lesions in both sense and antisense ODN-treated mice (data not shown). Interestingly, the magnitude of the increase in triglyceride level was similar to that in homozygous apo E-deficient mice (antisense 35 mg/dl versus homozygote 27 mg/dl). Thus, hepatic apo E may play an important role in triglyceride metabolism. Nevertheless, the increase in plasma cholesterol was not dramatic, probably due to the low amounts of ODN or the short period of the inhibitory effects of antisense ODN. The limitation of the present study to create a new animal model is clearly apparent considering the transient change in the targeted genes rather than permanent inhibition. Further modification of ODN such as the change of ODN backbone to increase the resistance to nuclease would resolve these issues.

Alternatively, *in vivo* systemic administration of antisense ODN against lipoprotein may be considered as a

gene therapy strategy for atherosclerosis. Lipoprotein (a) and apolipoprotein A-II are reported as independent risk factors for coronary artery disease (Davignon *et al.* 1988, Rubin *et al.* 1991, Abbey 1992, Wade *et al.* 1993, Morishita *et al.* 1994c). These lipoproteins are mainly synthesized in the liver, as for apo E. It is noteworthy that there is no pharmacotherapy to decrease such high risk lipoproteins selectively. We previously reported that ribozyme selectively induces cleavage of apo (a) without affecting plasminogen gene expression (Morishita *et al.* 1998). Together with the delivery system presented in this study, systemic administration of ribozyme against apo (a) may be useful to treat atherosclerosis induced by high lipoprotein (a). Systemic delivery of antisense or ribozyme by the HVJ-liposome method may provide the opportunity to treat such disease status. Moreover, this study addressed another potential problem, antigenicity of the viral vector system. We observed that the biological effects exerted by administration of antisense ODN were still reproduced following 8 repeated injections. Thus, it is unlikely that the HVJ-liposome method causes antigenicity. Indeed, we could not detect significant titers of antibody against HVJ after multiple repeated injections (Morishita *et al.* 2000). The low antigenicity of HVJ-liposome is consistent with a previous report (Hirano *et al.* 1998). Therefore, the simultaneous inhibition of double genes may be possible using the HVJ-liposome method, since we did not address this possibility in the present study. This character seems to be ideal for a future gene therapy strategy, although further characterization is needed. Overall, this study shows the feasibility of the *in vivo* antisense strategy to improve hyperlipoproteinemic metabolism.

In conclusion, systemic administration of antisense apo E ODN in the HVJ-liposome complex created hepatic apo E-deficient ('antigenic') mice which showed a transient increase in cholesterol and triglyceride. Repeated injection of antisense apo E ODN maintained apo E deficiency in mice over 3 months. Although apolipoprotein-deficient mice created by intravenous administration of antisense ODN seem to be a promising new animal model to help understand the role of apolipoprotein *in vivo* and develop new drug therapies targeting apolipoprotein, further development of ODN delivery methods and ODN backbone might be necessary.

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