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Suppression of diet-induced atherosclerosis in low density lipoprotein receptor knockout mice overexpressing lipoprotein lipase

(chylomicron remnants/transgenic mice/gene targeting/hypercholesterolemia/triglycerides)

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ABSTRACT **Lipoprotein lipase (LPL) is a key enzyme in the hydrolysis of triglyceride-rich lipoproteins. Conflicting results have been reported concerning its role in atherogenesis. To determine the effects of the overexpressed LPL on diet-induced atherosclerosis, we have generated low density lipoprotein receptor (LDLR) knockout mice that overexpressed human LPL transgene (LPL/LDLRKO) and compared their plasma lipoproteins and atherosclerosis with those in nonexpressing LDLR-knockout mice (LDLRKO). On a normal chow diet, LPL/LDLRKO mice showed marked suppression of mean plasma triglyceride levels (32 versus 236 mg/dl) and modest decrease in mean cholesterol levels (300 versus 386 mg/dl) as compared with LDLRKO mice. Larger lipoprotein particles of intermediate density lipoprotein (IDL)/LDL were selectively reduced in LPL/LDLRKO mice. On an atherogenic diet, both mice exhibited severe hypercholesterolemia. But, mean plasma cholesterol levels in LPL/LDLRKO mice were still suppressed as compared with that in LDLRKO mice (1357 versus 2187 mg/dl). Marked reduction in a larger subfraction of IDL/LDL, which conceivably corresponds to remnant lipoproteins, was observed in the LPL/LDLRKO mice. LDLRKO mice developed severe fatty streak lesions in the aortic sinus after feeding with the atherogenic diet for 8 weeks. In contrast, mean lesion area in the LPL/LDLRKO mice was 18-fold smaller than that in LDLRKO mice. We suggest that the altered lipoprotein profile, in particular the reduced level of remnant lipoproteins, is mainly responsible for the protection by LPL against atherosclerosis.**

Lipoprotein lipase (LPL) hydrolyzes triglycerides in plasma lipoproteins such as chylomicrons and very low density lipoproteins (VLDL), converting them to relatively cholesterol-rich atherogenic lipoproteins such as chylomicron remnants and low density lipoproteins (LDL), respectively (1, 2). On the other hand, LPL also plays a crucial role in the formation of anti-atherogenic high density lipoproteins (HDL) (3–5). In addition, LPL is essential for the clearance of apoprotein (apo) B-containing lipoproteins by the liver (6). These apparently opposing actions of LPL have generated controversies concerning its role in atherogenesis (7). Moreover, LPL is involved in atherogenesis not only by modulating plasma lipoprotein metabolism but also by functioning in the lesions *in situ* (8), making the issue more complicated.

Even clinical observations are conflicting. In the literature, few patients with LPL deficiency developed premature atherosclerosis, which favors a hypothesis that LPL is atherogenic (7). On the contrary, it has been shown that a heterozygous state for a LPL mutation is associated with premature atherosclerosis (9). Furthermore, pharmaceutical interventions that increased LPL activities were shown to protect against atherosclerosis both in humans (10) and animals (11). This circumstantial evidence supports the second hypothesis that LPL is anti-atherogenic.

In vitro studies have indicated that LPL stimulates cellular lipoprotein uptake through two distinct mechanisms. (*i*) Lipolysis causes conformational and compositional changes of lipoproteins, thereby increasing the affinity of lipoproteins to cellular lipoprotein receptors (12–14). (*ii*) LPL on the lipoprotein surfaces directly binds to the LDL receptor related protein/proteoglycans, a receptor for chylomicron remnants (bridging effects) (15–17). Atherogenecity of LPL would depend on which cells are more affected by the LPL-stimulated cellular lipoprotein uptake; liver cells or arterial wall cells. If it predominantly involves the liver cells, LPL will remove the atherogenic lipoproteins from the circulation, thus protecting against atherosclerosis. On the other hand, if its involvement in arterial wall cells is dominant over liver cells, LPL might enhance foam cell formation, thus promoting atherosclerosis. In support of the latter possibility, it has been shown that LPL is locally produced in the arterial walls (18–20).

Currently, murine models of atherosclerosis have been increasingly used for the study of atherogenic lipoproteins (21). It is known that a large variation exists in susceptibility to diet-induced atherosclerosis across strains (22). C57BL/6 mice, the most susceptible strain, shows the largest reduction of high density lipoprotein (HDL) cholesterol after feeding with an atherogenic diet, implying that HDL is an important determinant of atherogenicity (23). In support of this, it has been shown that transgenic mice overexpressing apo A-I are resistant to diet-induced atherosclerosis (24). On the other hand, mice overexpressing either apo A-II or cholesterol ester transfer protein developed severe diet-induced atherosclerosis (25, 26). Moreover, mice lacking apo E have turned out to develop extensive atherosclerosis (27, 28). Similarly, mice with targeted disruption of the LDL receptor gene (LDLRKO mice), an animal model of familial hypercholesterolemia, develop severe hypercholesterolemia and massive atherosclerosis when fed a high cholesterol diet (29). Because both mice accumulate remnant lipoproteins in the plasma, these lipoproteins are thought to mediate the atherogenecity of the mice.

Recently, we established lines of transgenic mice that overexpressed LPL, mainly in the heart, skeletal muscle, adipose tissue and, to a lesser extent, in other tissues including the aorta (30). The mice showed marked reduction in plasma triglyceride levels. To determine whether LPL reduces remnant lipopro-

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Abbreviations: LPL, lipoprotein lipase; apo, apoprotein; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; LDLRKO, low density lipoprotein receptor knockout.

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teins and protects against atherosclerosis, we created LDL-RKO mice that overexpress LPL (LPL/LDLRKO) by mating the LPL transgenic mice to LDLRKO mice. These animals, moreover, will be useful to estimate the significance of non-LDL receptor pathway for the LPL-stimulated lipoprotein uptake by the liver.

EXPERIMENTAL PROCEDURES

Materials and Methods. Blood was obtained from the retroorbital plexus after a 16-h fast (from 6 p.m. to 10 a.m.) as described (30, 31). Plasma cholesterol and triglyceride levels were determined enzymatically as described (30). Plasma lipoproteins were analyzed by high pressure liquid chromatography. Ten microliters of pooled fasting plasma was applied to a combined column system composed of TSK G3000SW + G5000SW (Tosoh, Tokyo) in sequence and eluted with 0.15 M NaCl/0.01% EDTA at a rate of 0.4 ml/min as described (30). For apolipoprotein analysis, the total lipoprotein fraction (d < 1.210 g/ml) was dialyzed against a solution containing 0.15 M NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride at pH 7.4 and delipidated with ethanol/ether (1:1). Apoproteins were subjected to 5–20% polyacrylamide gradient SDS/PAGE as described (29). The intensity of the bands were determined by densitometric analysis.

Mice. The LPL transgenic animals were created as described (30). To obtain LPL/LDLRKO, the heterozygous LPL transgenic mice were bred with the homozygous LDLRKO (32). The resultant offspring, which overexpressed LPL and were obligatorily heterozygous for the mutant LDL locus, were further bred to their littermates that lacked the LPL transgene. PCR was used to assign the genotype (33). Because genetic predisposition to atherosclerosis differs from strain to strain in mice, genetic background of the mice is important (22). The parental LDLRKO mice were F_2 hybrids between 129/Sv and C57BL/6, and the parental LPL transgenic mice were F_2 hybrids between C57BL/6 and DBA/2, which had been backcrossed into C57BL/6 three times. Therefore, 72%, 25%, and 3% of the genetic background of the mice was derived from the C57BL/6, 129/Sv, and DBA/2 strains, respectively. Both 129/Sv and DBA/2 are less susceptible to atherosclerosis than C57BL/6, which is known to be the most susceptible strain.

Diets. Two diets were used: (*i*) a normal chow diet (MF from Oriental Yeast, Osaka) and (*ii*) an atherogenic diet that contained 1.25% (wt/wt) cholesterol, 0.5% (wt/wt) sodium cholate, and 15% (wt/wt) saturated fat in the form of cocoa butter.

Lipoprotein Kinetics. After a 12-h fast, 400 μl of olive oil was given as a bolus dose into the stomachs of LDLRKO (n = 4) and LPL/LDLRKO (n = 4). Blood (50 μl) was collected serially at 0, 0.5, 1, 2, 4, 6, and 16 h after the oral fat loading. Plasma triglyceride levels were measured enzymatically. VLDL (d < 1.006 g/ml) and LDL (1.019 < d < 1.063 g/ml) were separated from the plasma of a normolipidemic healthy donor and pooled plasma of wild-type C57BL/6 mice (n = 18) after a 16-h fast, respectively, by ultracentrifugation (30). The lipoproteins were radioiodinated using the iodine monochloride method as described (20). A bolus dose of either ^{125}I-VLDL (5 μCi per animal) or ^{125}I-LDL (5 μCi per animal) was injected into three LDLRKO and three LPL/LDLRKO through the tail veins. The values at 1 min after injection were used as 100%. Plasma was ultracentrifuged in a 40–3 rotor (Beckman) at 38,000 rpm at 12°C for 16 h to separate the VLDL fraction. The apo B-associated radioactivities in VLDL and plasma were determined by the isopropanol precipitation method as described (30). The apo B-associated radioactivity in IDL + LDL was calculated by subtracting the VLDL value from the plasma value.

Lipid-Staining Lesion Area in the Mouse Aorta. The lesion areas were estimated according to a modified method of Paigen (34, 35). Briefly, the heart and attached aorta were resected, placed in 0.9% saline for 1 h, and incubated in 7% (wt/vol) formalin for 48 h. After 48-h in an OCT compound embedding medium, each sample was frozen on a cryostat, and beginning with the lower portions of atria, 10 μm cross sections were made. All sections were examined by microscopy without any stain. Four sections, each separated by 60 μm, were used to evaluate the lesions, two at the end of the aortic sinus and two at the junctional site of the sinus and ascending aorta (which were determined by the presence of three aortic valve cusps and a rounded aorta where the lesions were most prominent). Fatty steak lesions were stained using Oil Red O and hematoxylin/eosin. The lesion area of each section was estimated by weighing the excised pieces on enlarged photocopies of a photograph of the microscopic section. The mean lesion area per section per animal was calculated for each group.

RESULTS

When fed a normal chow, LPL/LDLRKO had plasma triglyceride and cholesterol levels that were 13.4% and 77.7% of those in LDLRKO, respectively (Table 1). Even after the atherogenic diet, the suppressive effects of the LPL transgene persisted for both plasma triglyceride and cholesterol levels. Although both animals developed severe hypercholesterolemia, LPL/LDLRKO exhibited significantly milder hypercholesterolemia as compared with LDLRKO. Upon high pressure liquid chromatography, triglycerides were distributed on the lipoproteins comprising VLDL (fraction a) through LDL (fraction c) in wild-type mice fed a normal chow (Fig. 1*A*). In LDLRKO fed a normal chow, the major fraction of triglycerides was distributed on a single peak in fraction b that eluted midway between VLDL (fraction a) and LDL (fraction c). The overexpression of LPL substantially reduced the size of this peak without changing the elution pattern. In LDLRKO fed a normal chow, cholesterol was distributed on both the IDL/LDL (fraction b + c) and HDL (fraction e) (Fig. 1*B*). The

Table 1. Human LPL mass in post-heparin plasma and plasma lipid levels in LPL/LDLRKO and LDLRKO mice fed either a normal chow or atherogenic diet

Diet	Mice	LPL mass (ng/ml)	Triglycerides	Cholesterol (mg/dl)	HDL cholesterol
Normal	LDLRKO	<20 (4)	236 ± 129 (19)	386 ± 90 (19)	96 ± 18 (9)
	LPL/LDLRKO	1740 ± 325 (4)	32 ± 19* (30)	300 ± 106† (30)	84 ± 17 (11)
Atherogenic	LDLRKO	ND	139 ± 49 (7)	2187 ± 243 (7)	74 ± 14 (3)
	LPL/LDLRKO	ND	13.4 ± 3.5‡ (9)	1357 ± 183‡ (9)	44 ± 12 (4)

Blood was collected from the mice before and 4 weeks after consumption of the atherogenic diet (30). Post-heparin plasma (fasting for 16 h) was taken 3 min after bolus injection of heparin (100 units/kg) into the tail vein. Human LPL mass was determined as described (14). Plasma triglyceride and cholesterol levels were determined enzymatically as described (30). HDL levels were determined from the HPLC profiles and total cholesterol levels. The mice were 6–10 weeks of age. Values are mean ± SD. Numbers of mice used for the studies are indicated in parentheses. ND, not determined.

*, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$ compared between the LDLRKO and LPL/LDLRKO (Student's t test).

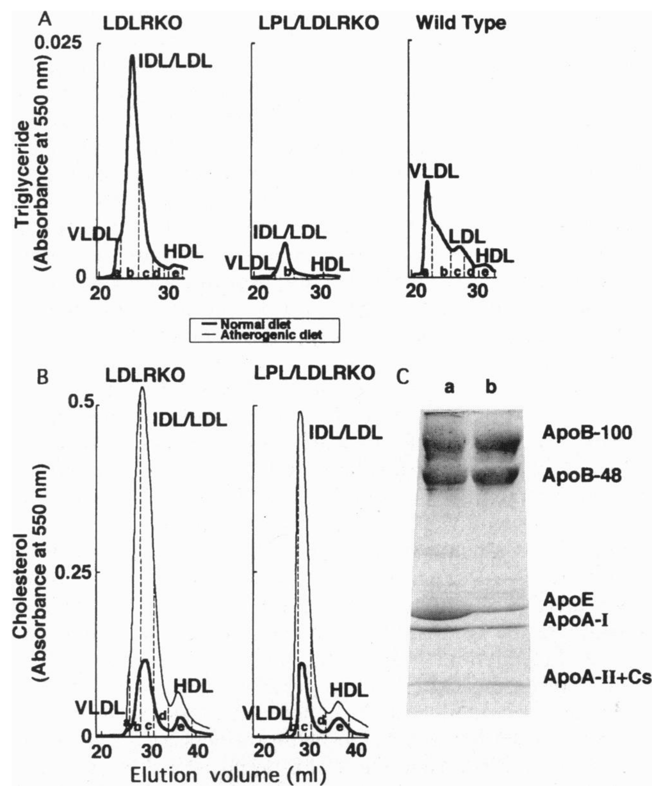

FIG. 1. Plasma lipoprotein analyses on LDLRKO and LPL/LDLRKO. (*A* and *B*) Plasma lipoprotein profiles separated by gel filtration chromatography. Triglyceride (*A*) and cholesterol (*B*) contents in the elution were measured enzymatically. The mice were fed either a normal diet (thick lines) or an atherogenic diet (thin lines). For comparison, elution profiles are further arbitrarily divided into five fractions (a–e). (*C*) SDS/PAGE analyses of plasma lipoproteins from the mice fed the atherogenic diet for 8 weeks. Lane a, LDLRKO; lane b, LPL/LDLRKO.

overexpression of LPL markedly reduced the cholesterol in IDL/LDL fraction (especially fraction b) without changing HDL significantly. When fed the atherogenic diet, LDLRKO showed an increase in IDL/LDL cholesterol level (Fig. 1*B*). Note that the LPL overproduction specifically reduced the larger particles of IDL/LDL (fraction b) resulting in a narrower IDL/LDL peak, which was comprised exclusively of smaller particles (fraction c). To semi-quantify the apoproteins, SDS/PAGE of lipoproteins was performed (Fig. 1*C*). Significant differences were not detectable in the amounts of either apo B-100 or apo B-48. On the other hand, apo E level was significantly reduced in LPL/LDLRKO as compared with LDLRKO. Apo AI was also moderately reduced.

To study the metabolism of the intestinal lipoproteins, a fat absorption test was performed (Fig. 2*A*). The area-under-the-curve of plasma triglyceride excursion in LPL/LDLRKO was suppressed by 70% as compared with that in LDLRKO. To determine the metabolism of the lipoproteins containing apo B-100, turnover studies using either ^{125}I-labeled VLDL (Fig. 2*B*) or ^{125}I-LDL (Fig. 2*C*) were performed. VLDL fractions of the injected ^{125}I-VLDL were disappeared from the plasma 50% faster in LPL/LDLRKO mice as compared with LDLRKO (16.2 versus 11.1 pools per day) (Fig. 2*B*). Concurrently, substantial amounts of the apo B-associated radioactivities in VLDL were converted to IDL/LDL. Similarly, the plasma clearance of ^{125}I-LDL in LPL/LDLRKO was identical to that in LDLRKO (Fig. 2*C*).

The mean lipid-staining lesion areas per mouse were 309,100 ± 216,200 μm^2 for LDLRKO and 17,100 ± 9,900 μm^2 for LPL/LDLRKO (Fig. 3*A*). Thus, the overexpression of LPL

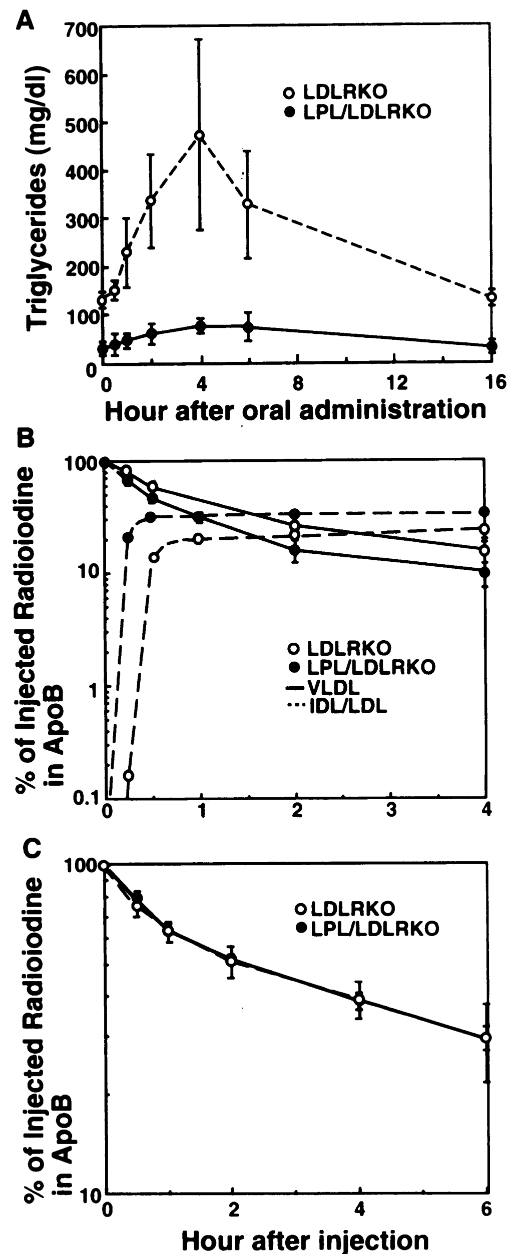

FIG. 2. Lipoprotein kinetics. (*A*) Plasma triglyceride levels after a bolus oral fat load. After a 12-h fast, 400 μl of olive oil was given as a bolus dose into the stomachs of LDLRKO (n = 4, ○) and LPL/LDLRKO (n = 4, ●). (*B*) Plasma clearance of ^{125}I-VLDL. A bolus dose of either ^{125}I-VLDL (5 μCi per animal) was injected into LDLRKO (n = 3, ○) and LPL/LDLRKO mice (n = 3, ●). The percent of radioactivities associated with apo B in VLDL (solid lines) and IDL + LDL (dashed lines) are indicated. (*C*) Plasma clearance of ^{125}I-LDL. A bolus dose of ^{125}I-LDL (5 μCi per animal) was injected into LDLRKO (n = 3, ○) and LPL/LDLRKO mice (n = 3, ●). The percent of radioactivities associated with total apo B were shown. Error bars indicate SD.

significantly reduced the lesion by 18-fold. Lesions typical of those seen in LDLRKO and LPL/LDLRKO are shown in Fig. 3*B*. LDLRKO mice had extensive foam cell lesions with necrotic acellular areas, whereas LPL/LDLRKO mice had only spotty fatty streaks. Since the degree of the suppressive effect of LPL on the diet-induced atherosclerosis was comparable to that of the reduction in either plasma triglyceride (10.3-fold) or apo E levels (≈10-fold) by LPL overproduction, the protective effects of LPL against atherosclerosis might be

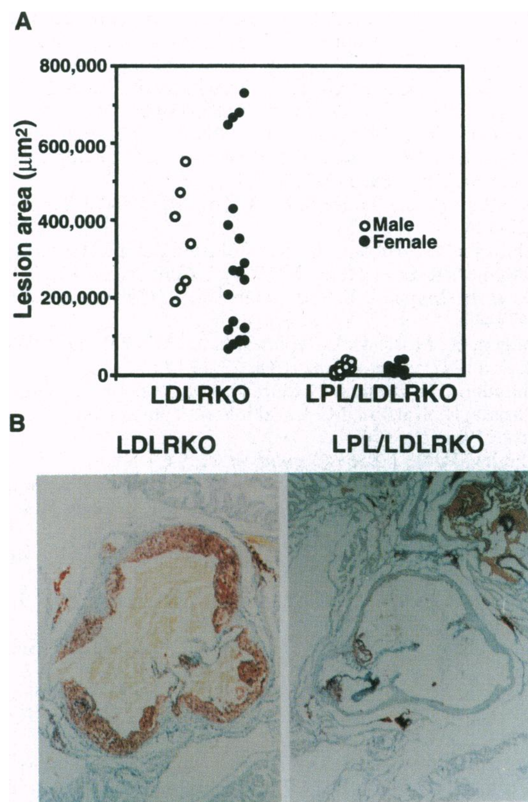

FIG. 3. Lipid-staining lesion area in the mouse aorta. (*A*) The mean lesion area per section is plotted in each of the LDLRKO and LPL/LDLRKO mice (○, males; ●: females). (*B*) Representative photographs at low magnification (50×) of cross sections of the proximal aorta from LDLRKO and LPL/LDLRKO.

mediated through the removal of the triglyceride-rich and apo E-rich remnants.

DISCUSSION

This study shows that overexpression of LPL suppressed atherosclerosis in LDL receptor-deficient mice, fed with an atherogenic diet, probably by converting the remnant lipoproteins to nonatherogenic smaller particles.

In our previous study (30), we found a slight elevation of cholesterol in HDL_2 of LPL transgenic mice fed a normal chow. In this study with the atherogenic diet, however, neither HDL cholesterol nor apo AI, both of which were found to be anti-atherogenic in the transgenic models (24), were increased in LPL/LDLRKO mice as compared with LDLRKO mice (Table 1, Fig. 1 *B* and *C*). Thus, it does not appear that the overexpressed LPL suppressed atherosclerosis by means of its increasing effects on HDL. Instead, it is more plausible that reduction of the remnants ("fraction c" in Fig. 1) mediates the anti-atherogenic effects of the overexpressed LPL. This particular subfraction of IDL/LDL conceivably corresponds to "remnants of triglyceride-rich lipoproteins" whose extreme atherogenecity has been demonstrated in clinical studies (36–38). Apolipoprotein compositional analyses suggested that this lipoprotein subfraction is rich in apo E (Fig. 1*C*). It seems that the overexpressed LPL converted them to the smaller, cholesterol-poor and apo E-poor particles, which are conceivably far less atherogenic than remnants. We speculate that surface components were pinched off from remnant particles by excessive lipolysis to such a degree that most of the apo E was lost. In this regard, it is noteworthy that the LDLRKO mice fed with a normal chow did not accumulate remnants and, as a result, did not develop such severe lesions as seen in those fed an atherogenic diet.

Because the plasma clearance of total apo B in either VLDL or LDL were not different between the LPL/LDLRKO and LDLRKO mice (Fig. 2 *B* and *C*), it is unlikely that the overexpressed LPL increased the net removal of the apo B-containing lipoproteins from the circulation, as is the case in mice with functional LDL receptor (30). The results of these turnover studies are consistent with the observation that the plasma levels of apo B-100 and apo B-48 were not altered (Fig. 1*C*), and further indicate that, in normal circumstances, LPL stimulates hepatic uptake of apo B-containing lipoproteins exclusively through LDL receptor. Apparently our results do not support the hypothesis that LPL bridges lipoproteins to the non-LDL receptors, LRP receptor related protein/proteoglycans, thereby facilitating hepatic removal of lipoproteins, as was suggested in *in vitro* studies (15–17). But it also remains possible that the LPL-stimulated cellular uptake is saturated by endogenous LPL and excess LPL does not further stimulate the lipoprotein uptake through non-LDL receptor pathway.

In addition to the effects of LPL on plasma lipoprotein profile as discussed above, local production of LPL in the arterial wall might be responsible for the suppression of atherosclerosis by the overexpressed LPL. Northern blot analysis demonstrated that the LPL transgene was expressed even in the aorta (30). Previous studies suggested that arterial LPL promotes atherosclerosis by facilitating either retention of lipoproteins (39) or foam cell formation (40, 41). If these hypotheses are true, the LPL/LDLRKO mice should have developed more extensive lesions than the LDLRKO mice. However, it was not the case. Local LPL might play a protecting role against atherosclerosis, presumably either by facilitating disposal of the lipoproteins from the extracellular spaces of the vessel wall or by converting lipoproteins to ones that have low affinity to the extracellular matrices and cell surfaces. Alternatively, the effects of LPL overexpressed throughout the body on the plasma lipoprotein profile might overwhelm those resulting from overexpression in arterial walls.

An effort has been made to correct the LDL receptor deficiency in human familial hypercholesterolemia (32, 42). Besides being an ideal modes of gene transfer, antibody formation against newly introduced proteins has emerged as a serious problem in gene therapy. Our current findings imply that such immunological problems intrinsic to familial hypercholesterolemia gene therapy can be circumvented by overexpression of LPL. Treatment of genetic diseases by manipulating the genes that are apparently irrelevant to the diseases seems of great promise.

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