Worsening of Diet-Induced Atherosclerosis in a New Model of Transgenic Rabbit Expressing the Human Plasma Phospholipid Transfer Protein


Objective—Plasma phospholipid transfer protein (PLTP) is involved in intravascular lipoprotein metabolism. PLTP is known to act through 2 main mechanisms: by remodeling high-density lipoproteins (HDL) and by increasing apolipoprotein (apo) B–containing lipoproteins. The aim of this study was to generate a new model of human PLTP transgenic (HuPLTPTg) rabbit and to determine whether PLTP expression modulates atherosclerosis in this species that, unlike humans and mice, displays naturally very low PLTP activity.

Methods and Results—In HuPLTPTg rabbits, the human PLTP cDNA was placed under the control of the human eF1-α gene promoter, resulting in a widespread tissue expression pattern and in increased plasma PLTP. The HuPLTPTg rabbits showed a significant increase in the cholesterol content of the plasma apoB-containing lipoprotein fractions, with a more severe trait when animals were fed a cholesterol-rich diet. In contrast, HDL cholesterol level was not modified in HuPLTPTg rabbits. Formation of aortic fatty streaks was increased in hypercholesterolemic HuPLTPTg animals as compared with nontransgenic littermates.

Conclusion—Human PLTP expression in HuPLTPTg rabbit worsens atherosclerosis as a result of increased levels of atherogenic apoB-containing lipoproteins but not of alterations in their antioxidative protection or in cholesterol content of plasma HDL. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: atherosclerosis ■ lipoproteins ■ metabolism ■ transgenic models

Human plasma contains 2 distinct lipid transfer proteins (cholesteryl ester transfer protein [CETP] and phospholipid transfer protein [PLTP]) that promote the rapid exchange of lipid species between circulating lipoproteins, as well as between circulating lipoproteins and cells. Although most reported data agree that the putative implication of CETP in lipoprotein metabolism and atherosclerotic process mostly concerns the net transfer of cholesteryl esters from antiatherogenic high-density lipoproteins (HDL) to potentially atherogenic apolipoprotein (apo) B–containing lipoproteins,1 the precise function of PLTP and its consequences in terms of atherogenesis remain a matter of debate. PLTP was identified initially for its ability to promote the transfer of phospholipids from triglyceride-rich lipoproteins to HDL in the postprandial phase.2 Subsequent studies reported that PLTP is also a key player in the HDL conversion process, leading to the emergence of large and small HDL products.3,4 Recently, PLTP in human plasma was shown to reside mainly on lipid-poor complexes in association with proteins linked to immunity and inflammation, suggesting that the biological function of PLTP is rather complex in nature.5 In support of this view, PLTP expression in the mouse was found to increase the production of apoB-containing lipoproteins by the liver6 and intestine,7 to decrease very low density lipoprotein (VLDL) catabolism,8 to reduce the antioxidative protection of atherogenic lipoproteins,9,10 to trigger inflammation,11 and to promote the association of lipopolysaccharides with circulating lipoproteins.12 All these properties have long been shown to influence the atherosclerotic process, whether acting individually or in a combined manner.

High plasma PLTP concentrations were earlier reported in diabetic patients, who are known to be at high risk of atherosclerosis and cardiovascular disease.13 Accordingly, the level of plasma PLTP activity was found to have an independent predictive value for carotid intima-media thickness and coronary artery disease in high-risk patients.14,15 In a cohort of Chinese patients with or without coronary artery disease, plasma PLTP activity was recently shown to associate nega-
with plasma HDL and apoAI levels and positively with plasma triglycerides, apoB levels, and apoE levels. In a large cross-sectional study, high PLTP in diabetics was associated with significant changes in HDL size distribution, as well as with an increase in apoB-containing lipoprotein level. In recent genome-wide analyses, a number of loci were found to associate with HDL cholesterol and triglycerides; among them, 1 was localized to the PLTP gene. In a hyperalphalipoproteinemic human population, carriers of the rs2294213 allele of the PLTP gene were found to combine markedly elevated VLDL cholesterol, modestly elevated triglycerides, and modestly decreased HDL cholesterol. In patients with carotid artery disease, single-nucleotide polymorphisms associated with PLTP activity were found to predict variation in low-density lipoprotein (LDL) cholesterol but not in HDL cholesterol. By combining several case-control studies, a gene score based on PLTP variants with low PLTP transcription and activity was found to associate with changes in the size and number of HDL, as well as with a decreased risk of cardiovascular diseases. Overall, and although human studies support PLTP as a cardiometabolic risk factor, it remains unclear at this stage whether the increased susceptibility to atherosclerosis related to the high PLTP trait mainly concerns decreased HDL concentrations, increased apoB-containing lipoprotein level, decreased antioxidative protection of lipoproteins, or a combination of these abnormalities.

Elucidation of the relative contribution of distinct PLTP functions to lipoprotein metabolism and atherogenesis in earlier animal studies might have been blurred in part because they were conducted mostly, if not only, in the mouse model. Indeed, unlike humans, the mouse is characterized by undetectable plasma CETP activity, high HDL cholesterol, low apoB-containing lipoproteins, and low susceptibility to atherosclerosis. Even with the recent use of mouse models combining hyperlipidemia and elevated susceptibility to atherosclerosis, the overwhelming accumulation of apoB-containing lipoproteins and elevated expression of a human PLTP transgene in the mouse CETP-deficient background may have been significant drawbacks.

The rabbit model displays several advantages over rats and mice. Unlike rats and mice, but like humans, the rabbit is an atherosusceptible species that combines high CETP activity and an accumulation of plasma apoB-containing lipoproteins when fed a cholesterol-enriched diet. In addition, the rabbit displays the lowest plasma PLTP activity ever reported among vertebrate species, whereas the mouse is the animal species with the highest PLTP activity. Thus, although a clear phenotype after additive PLTP transgenesis in the mouse arose in those animals only with pronounced or sufficient increases in PLTP expression level, it can be postulated that moderate expression of human PLTP in the low-PLTP rabbit model might be sufficient to produce a significant alteration in lipoprotein metabolism. The first transgenic rabbit model expressing the human PLTP gene and with moderate increments in plasma phospholipid transfer activity was generated in the present study, with the human PLTP transgene placed under the control of the ubiquitous human eF1-α gene promoter. Consequences on lipoprotein profile and atherogenesis were addressed.

Methods

A Supplemental Methods section is available online at http://atvb.ahajournals.org.

Recombinant human and rabbit PLTP were produced by transfection in human colorectal carcinoma HCT116 cells. To generate human PLTP transgenic (HuPLTPtg) New Zealand White rabbits, the human PLTP cDNA was inserted into the pMD3 vector, which is known to produce a ubiquitous expression of transgenes in the rabbit. PLTP activity was measured using a commercially available fluorescence activity assay (Roar Biomedical Inc, New York, NY) or a radioactive transfer assay. Lipoproteins were separated by ultracentrifugation, gradient gel electrophoresis, or fast protein liquid chromatography. For the atherosclerosis study, the rabbits were fed a 1% cholesterol-enriched diet, and atherosclerotic lesion areas were determined by en face observations.

Results

Characterization of Rabbit PLTP

Among 14 vertebrate species, the rabbit was found to display one of the lowest plasma phospholipid transfer activities. Rabbit PLTP cDNA has a high, 86.1% homology with human PLTP cDNA, despite duplication of the REVIDKN sequence in the C terminus of the rabbit protein. The 7-amino-acid insertion was confirmed in the present study, but here, as in humans, baboons, pigs, mice, and cows, with an isoleucine instead of a methionine at position 395 of the rabbit protein (GenBank AF382033). To work out whether low phospholipid transfer activity in rabbit plasma might relate to dysfunctional PLTP, recombinant rabbit and human mature PLTPs were obtained by transient transfection in human HCT116 cells. In both cases, similar amounts of a unique 80-kDa protein were detected by Western blot in the culture media of transfected cells but not in that of mock-transfected cells (Figure 1A). PLTP of either source showed similar specific phospholipid transfer activities (Figure 1B).

Generation of PLTP-Transgenic Rabbits

We generated a new model of transgenic rabbit in which the human PLTP gene was placed under the control of the human eF1-α gene promoter (HuPLTPtg rabbits) (Figure 2A). Three founders (HuPLTP2, HuPLTP3, and HuPLTP4) were identified using polymerase chain reaction (Figure 2B). Human PLTP is a secreted protein, and its expression produced a significant increase in the plasma level of human PLTP in the HuPLTP3 rabbit founder as assessed by Western blot analysis with anti-human PLTP antibodies (Figure 2C). Accordingly, and as compared with wild-type (WT) controls, human PLTP transgene expression produced a marked increase in phospholipid transfer activity in the plasma of the HuPLTP3 founder, with a moderate but still significant increase in the HuPLTP4 founder (Figure 2D). As expected, and as compared with WT littermates, significant increases in the level of PLTP protein (Figure 3A) and phospholipid transfer rate (Figure 3B) were measured in the plasma of heterozygotes from the HuPLTP3 F1 generation, with transfer values lying between those found in WT rabbit plasma and those found in normolipidemic human plasma by using a fluorimetric assay (Figure 3B). By using a radioassay with...
radiolabeled liposomes as donors and isolated HDL as acceptors, phospholipid transfer rate was again higher in HuPLTPTg rabbits than in WT rabbits, here with similar transfer values in normolipidemic human plasma and HuPLTPTg rabbit plasma (Figure 3C). In good agreement with earlier observations in Chinchilla Bastard rabbits, expression of the rabbit PLTP gene was not confined to a given tissue in New Zealand White rabbits, and pancreas and adipose tissue expressed elevated amounts of rabbit PLTP mRNA (Figure 3D). Substantial levels of human PLTP mRNAs were detected in liver, adipose tissue, pancreas, kidney, lung, brain, heart, and spleen of HuPLTPTg rabbits, exceeding in all cases the level of endogenous rabbit PLTP mRNA as indicated by the human to rabbit PLTP mRNA ratio (Figure 3D).

**PLTP-Mediated Changes in Plasma Lipid Levels and Lipoprotein Distribution in HuPLTPTg Rabbits on a Chow Diet**

As shown in Figure 4A, quantitative analysis of plasma parameters in HuPLTPTg rabbits fed a chow diet revealed higher cholesterol content of non-HDL, apoB-containing lipoproteins as compared with WT littermates, reaching the statistical significance level for the male subgroup only. In contrast to non-HDL cholesterol, PLTP expression produced no significant changes in other plasma parameters, including HDL cholesterol, phospholipids, triglycerides, apoAI, and apoB (Figure 4A). C-reactive protein (CRP) levels did not differ in HuPLTPTg rabbits and their WT counterparts (Figure 4A). Fast protein liquid chromatography analysis of plasma from HuPLTPTg rabbits fed a chow diet revealed that PLTP expression was accompanied by 2 types of alterations as compared with the lipoprotein spectrum of WT controls. First, it produced a slight shift of the HDL peak toward the large size range (with a moderate, \(~1.5\)-nm increment in the mean diameter of plasma HDL as determined by polyacrylamide gradient gel electrophoresis; Supplemental Figure I) but with no change in net cholesterol and triglyceride content of the HDL-containing fractions (Figure 4B and 4C). Second, enhanced PLTP expression tended to increase the cholesterol and triglyceride contents of the LDL fraction, with the

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**Figure 1.** Similar specific phospholipid transfer activities of human and rabbit PLTP. A, The production of human and rabbit PLTP by transfected HCT116 cells was assessed by Western blot analysis of cell supernatants using an anti-flag M2 antibody (1, mock-transfected cells; 2, human PLTP; 3, rabbit PLTP). B, Concentration-dependent phospholipid transfer activities obtained with recombinant human and rabbit PLTP (fluorescent assay; see Methods).

**Figure 2.** Generation of HuPLTPTg rabbit lines. A, Structure of the human PLTP gene construct. The pMd3 vector contains 2 copies of the 5'HS4 insulator from the chicken \(\beta\)-globin locus; the promoter and first exon of the human \(\alpha\)-globin gene; a region of the MMTV promoter containing enhancers; the second intron of the rabibit \(\beta\)-globin gene; the HTLV-1 IRES, which contains a strong enhancer; the human PLTP cDNA; and the human GH gene terminator.47 B, Polymerase chain reaction identification of transgenic (HuPLTP) and WT (Neg) rabbits. Rabbit \(\beta\)-globin was used as positive control. C, Western blot analysis of human PLTP in the plasma of rabbits. D, PLTP activity in plasma of F0 HuPLTPTg rabbits and WT rabbits. Error bars indicate SEM.
statistical significance being reached only for the fast protein liquid chromatography/LDL-containing fractions numbered 15 to 20 (Figure 4B and 4C). In further support of LDL changes in chow-fed animals, HuPLTPTg LDL were of larger size than WT LDL on polyacrylamide gradient gels, and the cholesterol to protein ratio of isolated LDL was significantly higher in HuPLTPTg than in WT mice (Figure 4D and 4E). Percentage composition analysis of individual lipoprotein classes (including VLDL, IDL/LDL, and HDL) did not reveal marked differences between HuPLTPTg and WT rabbits, with the exception of significant increases in HDL free cholesterol content and HDL free cholesterol to cholesteryl ester ratio in HuPLTPTg rabbits (Supplemental Table I). The latter point is consistent with earlier mouse observations by Jiang et al., and it was proposed to relate directly to the transfer activity of PLTP rather than to putative alterations in LCAT activity, which in the present study was identical in HuPLTPTg and WT rabbits whether they were fed the standard chow (0.607 ± 0.022 versus 0.535 ± 0.027 AU, respectively; not significant) or the hypercholesterolemic diet (0.176 ± 0.010 versus 0.160 ± 0.010 AU, respectively; not significant). Finally, the occurrence of HDL enlargement in the absence of alterations in the relative composition of the HDL core is consistent with the previously described HDL fusion process mediated by PLTP.

Cholesterol Feeding Exacerbates PLTP-Mediated Changes in ApoB-Containing Lipoproteins of HuPLTPTg Rabbits

Male rabbits were fed a cholesterol-enriched diet for 4 and 8 weeks. As expected, this produced a major, up to 10-fold increase in plasma cholesterol concentration with a moderate, approximately 2-fold increase in plasma triglycerides as compared with animal counterparts fed the standard chow (Figure 5A). After 4 weeks of the high-cholesterol diet, PLTP expression produced 2-fold increases in plasma total chole-
terol and triglycerides in HuPLTPTg as compared with WT rabbits. Higher concentrations of non-HDL cholesterol were still observed in HuPLTPTg rabbits when fed the hypercholesterolemic diet for 8 weeks, and at this later time point approximately 2-fold increases in plasma triglycerides were observed in both WT and HuPLTPTg rabbits as compared with littermates fed the standard chow (Figures 4A and 5A). Again, and as observed above in chow-fed animals, CRP levels did not differ in HuPLTPTg and WT rabbits fed the cholesterol-enriched diet for 8 weeks (Figure 5A). Only non-HDL lipoproteins accounted for magnified hypercholesterolemia in the transgenic animals (Figure 5), and the cholesterol content of the ultracentrifugally isolated HDL remained constant in HuPLTPTg and WT rabbits whether they were fed the cholesterol-enriched diet for 4 or 8 weeks (Figure 5A).

Whereas accumulation of cholesterol was restricted to the fast protein liquid chromatography fractions in the LDL size range in chow-fed HuPLTPTg rabbits (Figure 5B), significant increases in the cholesterol contents of both VLDL- and LDL-containing fractions were observed in cholesterol-fed HuPLTPTg rabbits as compared with their WT counterparts (Figure 5B). In contrast to rabbits fed the chow diet, less than 20% of total plasma cholesterol was associated with HuPLTPTg HDL and WT HDL after high-cholesterol feeding. As shown in Supplemental Figure II, phospholipid transfer activity measured with the radioassay was 3-fold higher in the plasma of HuPLTPTg rabbits compared with WT rabbits, with consistent observations whether animals were fed the standard chow or the transgenic diet. Percentage composition analysis of individual lipoprotein classes (including VLDL, IDL/LDL, and HDL) did not reveal clear differences whether cholesterol-fed rabbits expressed the human PLTP transgene or not. In further support of an increased number, rather than cholesterol enrichment of individual lipoprotein particles accounting for the more severe hypercholesterolemic trait in HuPLTPTg rabbits, the approximately 50% mean increment in non-HDL cholesterol

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Figure 4. Plasma lipoprotein profiles in HuPLTPTg and WT rabbits fed the chow diet. (A) Plasma lipid and apolipoprotein parameters and CRP concentration in WT and HuPLTPTg rabbits fed a chow diet. All values are mean±SD (mmol/L, except apolipoproteins values in g/L). HDL-C indicates HDL cholesterol; LDL-C, LDL cholesterol. *P<0.05 versus WT counterparts (t test). Plasmas from WT and transgenic rabbits were passed through a Superose 6 HR column, and total cholesterol (B) and triglyceride (C) content of individual fractions was determined. Values are mean±SEM. D, Total plasma lipoproteins (d<1.21 g/mL fraction) were subjected to electrophoresis on native 15 to 250 g/L polyacrylamide gradients gels, and LDL size distribution was determined by comparison with protein standards (χ² test). E, The cholesterol and protein contents of LDL isolated as the 1.006<d<1.063 fraction were determined as described in Materials and Methods. *P<0.05 versus WT counterparts (t test).
in those animals as compared with their WT counterparts was accompanied by a similar, approximately 50% mean increment in plasma apoB concentration (Figure 5A).

High ApoB-Containing Lipoprotein Levels Are Associated With Increased Atherosclerosis Susceptibility in HuPLTPTg Rabbits

Male HuPLTPTg and WT rabbits were fed the high-cholesterol diet for 8 weeks, and the extent of fatty streaks was quantified on en face mounts of the aortic arch and descending aorta after Oil Red O staining. As shown in Figure 6, computerized morphometry revealed that the area of atherosclerotic lesion on the stained aorta was significantly increased in HuPLTPTg rabbits as compared with WT controls \( (P<0.05) \). A significant impact of PLTP expression was even more obvious when distinguishing between transgenic animals with supramedian and inframedian plasma PLTP activity values (Figure 6B). Indeed, HuPLTPTg rabbits with supramedian PLTP activity displayed 92% and 57% higher lesion area as compared with both WT and HuPLTPTg rabbits with inframedian PLTP activity, respectively \( (P<0.05 \text{ in both cases; Figure 6B}) \). As shown in Supplemental Figure III, immunostaining of aortic lesions revealed accumulation of macrophages in the subendothelial cells, which was associated with thickening of arterial intima in both WT and HuPLTPTg rabbits. No evidence for advanced lesions, for instance with smooth muscle cell recruitment in the intima, could be brought in either WT or HuPLTPTg rabbits (Supplemental Figure III). Lesion features in rabbits fed the cholesterol-enriched diet for 8 weeks are consistent with earlier data in rabbits fed a 1% cholesterol-containing diet for 10 weeks.\(^{31}\)

Among both WT and HuPLTPTg rabbits, significant positive correlations between lesion size and VLDL + LDL cholesterol levels were observed \( (\rho=0.562, n=24, P=0.0071 \text{ in HuPLTPTg rabbits}; \rho=0.542, n=23, P=0.011 \text{ in WT rabbits}) \). Plasma PLTP activity correlated positively with lesion size in the HuPLTPTg rabbits only \( (\rho=0.530, P=0.011) \), not in the WT rabbits \( (r=0.185, \text{not significant}) \). This suggests that plasma PLTP activity level became high enough in the HuPLTPTg rabbits to behave as a significant determinant of the extent of aortic lesions, but it was insufficient in the WT rabbits, in which plasma PLTP activity level is known to be
in the low range as compared with other vertebrate species.

Finally, neither plasma HDL cholesterol levels nor the \( \alpha \)-tocopherol content of lipoproteins correlated with the lesion area in either group (Supplemental Figure IV).

**Discussion**

PLTP is a member of the lipid transfer/lipopolysaccharide binding protein gene family and can be secreted by a large variety of tissues. It has the ability to bind and transfer a number of amphipathic compounds, including phospholipids, unesterified cholesterol, diacylglycerides, vitamin E, and lipopolysaccharides.\(^1\)\(^,\)\(^3\)\(^,\)\(^4\)\(^,\)\(^11\) It is shown here that rabbit PLTP is as effective as human PLTP in transferring phospholipids in vitro, but with a lower phospholipid transfer rate in rabbit plasma. Unlike the mouse, the rabbit combines high plasma CETP activity, substantial levels of apoB-containing lipoproteins that are similar to those found in humans, production of apoB100-containing VLDL by the liver, and high susceptibility to diet-induced hypercholesterolemia and atherosclerosis.\(^24\)\(^,\)\(^25\) Although this makes the rabbit a highly relevant model for additive PLTP transgenesis to explore the pathophysiological relevance of PLTP functions, it should be borne in mind that rabbit also displays a number of differences from humans (e.g., hepatic lipase and apoAII deficiency) that might have an impact on results obtained in this species.

In the present study, the human PLTP gene was placed under the control of the ubiquitous human \( \alpha \)-1-antitrypsin gene promoter, which made it possible (1) to obtain an expression level of the transgene with a construct similar to the one successfully used by our group to generate transgenic rabbits expressing the enhanced green fluorescent protein,\(^28\) (2) to reproduce a widespread tissue distribution of PLTP as observed in WT animals,\(^29\) (3) to minimize the tissue-specific effects that can occur locally when PLTP is produced exclusively at 1 given site,\(^32\) and (4) to investigate the systemic effect of circulating PLTP in animals with normolipidemia or diet-induced hypercholesterolemia. Although increases in the PLTP protein level paralleled increments of plasma phospholipid transfer activity (Figure 3), additional studies will be needed to determine in transgenic rabbits the contribution of the human PLTP transgene expression to the low versus high active forms of circulating human PLTP.\(^33\)\(^,\)\(^34\) The HuPLTPTg rabbits provided a unique model to address in the present study the crucial yet controversial implication of PLTP in atherosclerosis.

Earlier in vitro studies reported that PLTP has the ability to enlarge HDL, with no effect on LDL size and with inconstant production of minor small HDL subpopulations.\(^3\)\(^,\)\(^4\) Hu-PLTPTg rabbits here displayed moderate HDL enlargement as compared with WT rabbits, but with no concomitant increase in subpopulations of smaller particles. Again, this occurred in the absence of significant changes in plasma HDL cholesterol levels. These observations in HuPLTPTg rabbits clarified the role of PLTP in the HDL conversion process in a species whose lipoprotein parameters are closer to humans than are those of mice. In genetically engineered mice, contradictory observations were reported with either no change, a PLTP-mediated increase, or a PLTP-mediated decrease in HDL particle size.\(^27\)\(^,\)\(^35\)\(^--\)\(^39\) In contrast, the present rabbit data resemble some human observations in which increased PLTP activity was associated with enlarged HDL.\(^17\)\(^,\)\(^40\) It is likely to result from the previously described conversion process involving the fusion of HDL particles of intermediate size.\(^30\) Unlike HDL cholesterol, apoB-containing lipoprotein cholesterol underwent a clear rise in HuPLTPTg rabbits, and there was a much stronger effect when the animals were shifted from the standard chow to the high-cholesterol diet. Again, these data are in contrast with mouse data, in which the plasma HDL fraction predominates.\(^27\)\(^,\)\(^36\)--\(^39\) In the HuPLTPTg rabbit model, the PLTP transgene was placed under the control of the ubiquitous human \( \alpha \)-1-antitrypsin gene promoter, which is not known to respond to dietary cholesterol. Accordingly, PLTP transfer rates were identical in HuPLTPTg rabbits whether they were fed the standard chow or the cholesterol-enriched diet. Thus, the shift from a dual effect on HDL enlargement and moderately increased LDL cholesterol in chow-fed HuPLTPTg rabbits to a severe and isolated increase in VLDL+LDL cholesterol in cholesterol-fed animals resulted from the lipoprotein status, not from putative lipid-dependent regulation of PLTP transgene expression. In other words, the present study in both chow-fed/normolipidemic and cholesterol-fed/hyperlipidemic HuPLTPTg rabbits indicates that the impact of PLTP expression on apoB-containing lipoproteins versus HDL would be dependent mainly on the predominance of one lipoprotein class over the other. First, when HDL cholesterol predominates, PLTP has a prominent impact on HDL cholesterol and size. In this context, and as observed earlier in mice, the expression level of PLTP would actually determine the trend, i.e., increases in HDL cholesterol and size with moderate increases in PLTP versus decreases in HDL cholesterol and size with high increases in PLTP. Second (and in the case of balanced HDL and apoB-containing lipoprotein pools, as was the case here in the normallipidemic chow-fed HuPLTPTg rabbits), moderate PLTP expression produces concomitant but limited changes in both HDL (which differ only in size) and apoB-containing lipoproteins (with increased cholesterol content restricted to the LDL fraction). Finally, when VLDL and LDL predominate, as here in hypercholesterolemic rabbits, PLTP expression accentuates cholesterol accumulation in these lipoproteins only, with no effect on HDL. PLTP-mediated alterations of apoB-containing lipoproteins and not of HDL should then be considered the major concern in high-risk, dyslipidemic patients with high PLTP expression level (as is known to occur in type 2 diabetes\(^13\)). This view is illustrated by recent human studies in low HDL/dyslipidemic patients in whom PLTP activity was found to correlate more strongly with VLDL/LDL, than with HDL parameters.\(^15\)\(^,\)\(^18\)\(^,\)\(^19\)\(^,\)\(^31\)\(^,\)\(^32\) Finally, and on the basis of recent mouse observations that reported that PLTP can shift toward apoB-containing lipoproteins when HDL are scarce,\(^33\) higher amounts of PLTP are expected to associate with apoB-containing lipoproteins at the expense of HDL in cholesterol-fed rabbits as compared with animals fed the standard chow.

In an earlier study, Jiang et al\(^6\) demonstrated in several hyperlipidemic mouse models that PLTP deficiency is associated with a marked reduction in atherosclerotic lesions.
Decreased plasma levels of apoB-containing lipoproteins were proposed as the major antiatherogenic mechanism involved. However, they were accompanied by changes in the plasma concentration of HDL, and PLTP deficiency produced no alteration in the plasma level of apoB-containing lipoproteins in LDL receptor knockout mice. In subsequent studies, atherosclerosis lesions were found to be increased in the proximal aorta of AAV-PLTP-injected mice with a 2-fold increase in plasma PLTP activity; this, however, with no alteration in the cholesterol content of VLDL/LDL but significant decreases in the vitamin E content and antioxidative protection of apoB-containing lipoproteins. Moreover, increases in plasma PLTP and in susceptibility to atherosclerosis in LDL receptor \(^{+/+}/HuCETPTg\)/HuPLPTg mice as compared with LDL receptor \(^{+/−}/HuCETPTg\)/HuPLPTg mice were unexpectedly associated with lowering of apoB-containing lipoproteins. In this context, the present observations in cholesterol-fed HuPLPTg rabbits bring new insights. It is now demonstrated that moderate expression of human PLTP in the rabbit model with diet-induced hypercholesterolemia is associated with increased fatty streak formation in the aorta, with up to a 2-fold increase in lesion size of HuPLPTg rabbits as compared with WT controls. More importantly, it is clearly shown to be independent of HDL changes, which did not occur in HuPLPTg rabbits. Correlation analysis confirmed that the size of the lesions in hypercholesterolemic HuPLPTg rabbits was related to the VLDL+LDL cholesterol content and neither to the HDL level nor to the vitamin E content/antioxidant protection of apoB-containing lipoproteins (ie, 2 PLTP-modifiable parameters). Two main points should be considered when addressing the putative molecular mechanism that would account for the more severe hypercholesterolemic trait of cholesterol-fed HuPLPTg rabbits as compared with cholesterol-fed WT rabbits. First, when PLTP was found to delay the catabolism of apoB-containing lipoproteins in some of the earlier mouse studies, it was accompanied by both the accumulation of triglyceride-rich lipoproteins in plasma and marked alterations in LDL structure and composition. It was not the case in the present study after 8 weeks of dietary manipulation because in cholesterol-fed HuPLPTg rabbits the plasma triglyceride concentration, as well as the overall structure and composition of circulating apoB-containing particles, remained unchanged despite a 50% increment in LDL particle number as compared with cholesterol-fed WT rabbits. In contrast, when PLTP expression was found in several mouse models to act mainly through increased lipidation and secretion of apoB-containing lipoproteins in the liver, it was not associated with significant alterations in the relative composition of apoB-containing lipoproteins secreted by the liver but only in their production rate. The latter view fits very well with composition analyses of the present study made in hypercholesterolemic HuPLPTg rabbits and suggests that the molecular mechanism accounting for increased circulating apoB-containing lipoprotein levels and atherosclerosis in these animals could rely mainly on increased production of the atherogenic lipoproteins.

In conclusion, the present study demonstrates that increased VLDL+LDL cholesterol primarily accounts for the proatherogenic effect of PLTP in hypercholesterolemic rabbits. It provides new support for the relevance of PLTP inhibition in the prevention and treatment of atherosclerosis when plasma cholesterol distribution is shifted from HDL toward the proatherogenic apoB-containing lipoproteins. Although it is promising, the PLTP inhibition strategy should be considered with caution because the inhibition of PLTP activity might at the same time modify the formation of protein complexes implicated in host defenses and slow down lipopolysaccharide detoxification.

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**Disclosures**

None.

**References**


PLTP and Atherosclerosis in Transgenic Rabbits

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SUPPLEMENTAL MATERIAL

Production of recombinant human and rabbit PLTP by transfection in HCT116 cells

The cDNA coding for the mature, plasma rabbit protein (from aminoacid 18 to the C-terminal end of the protein) was obtained by PCR using R7 (forward) 5’-GCTAAAGCTTGAGATCCCAGGCT-3’ and R8 (reverse) 5’-CGTGGATCCTGAATGGCAGC-3’ primers that contained Hind III and BamHI restriction sites, respectively. Human and rabbit PLTP cDNAs were inserted into a P3XFLAG-CMV-8™ expression vector (Sigma) which was used to obtain transient expression and extracellular secretion of N-terminal 3X-FLAG/PLTP fusion products in human colorectal carcinoma HCT116 cells. One day before transfection, the cells were seeded onto 25 cm² dishes, and they were transfected with 2 μg of plasmid DNA using the Lipofectamine plus reagent™ (Invitrogen/Life Technologies) following the general protocol provided by the manufacturer. The transfected cells were incubated in serum free Opti-MEM (Invitrogen/Life Technologies) for 24 to 72 hours. Supernatants were then collected, spun at 1500 g for 10 minutes to remove detached cells, and they were finally concentrated in 30 K Ultrafree-4™ centrifugal filter Units (Millipore).

Western blot analyses

Fusion proteins from concentrated cell supernatants (10 μl) were separated on 8% SDS polyacrylamide gradient gels prior to transfer to nitrocellulose membranes. The resulting blots were developed with an anti-FLAG M2 antibody (Sigma) following the instructions of the manufacturer. Finally the blots were revealed with a chemiluminescent system (ECL™). The size of the secreted proteins was determined by comparison with biotinylated molecular weight markers (Sigma). Western blot analysis of PLTP in rabbit plasmas was performed as previously described by using a monoclonal antibody for PLTP (clone JH59 kindly provided by C. Ehnholm and M. Jauhiainen).

Quantification of recombinant PLTP

Recombinant PLTP in samples was quantitated by an immunoassay using recombinant 3XFLAG™ Bacterial Alkaline Phosphatase (BAP) protein as a standard. Briefly, serial dilutions of each sample were spotted on a nitrocellulose membrane. The dots were developed by anti-FLAG M2 antibodies and analysis was conducted as described above. Intensities of individual spots were determined by densitometric scanning of the films, and the concentration of recombinant PLTP in each sample was calculated by comparison with the calibration curve constructed with serial dilutions of the 3XFLAG™ BAP control protein.

Generation of HuPLTPTg rabbits

To generate HuPLTPTg rabbits, New-Zealand White rabbit does (A-1077, INRA, France) were superovulated by injections of porcine follicle-stimulating hormone. They were mated with males of the
same genetic background. Embryos were collected 17 hours later and male pronuclei were microinjected with the PLTP expression vector. The embryos were then transferred to pseudopregnant females. For gene construction, the human PLTP cDNA was inserted into the pMd3 vector described in Figure 2. The pMd3 vector has been shown in a previous study to produce an ubiquitous expression of transgenes in the rabbit. The expression of PLTP cDNA in the host organism was governed by the ubiquitous human eF1-α gene promoter (extending from nucleotides −1412 to +1 relative to the transcription start site of the eF1-α gene). The presence of the transgene was revealed by PCR on ear extracted DNA using hGH5’ (5’-AAGTTCGACACAAACTCACA-3’) and hGH3’bis (5’-AGCAATTTGGAGGCCAAGG-3’) primers. Two males of four transgenic founders expressed significant levels of plasma PLTP activity. The male with the highest expression level was crossbred with females of the standard New Zealand White line to provide the F2 animals used for analysis. HuPLTPTg and New-Zealand WT rabbits were fed a standard chow diet, and had free access to water and food. For the atherosclerosis study, the rabbits were fed a 1 % cholesterol-enriched diet for 8 weeks and had free access to water and food.

**Real time PCR analysis**

Total RNA was extracted from frozen tissues using Trizol reagent (Invitrogen). 100 to 300 ng of RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase, Random Primers and RNaseOUT inhibitor (Invitrogen). cDNA were quantified by real time PCR using a SYBR® Green Real-time PCR kit (Invitrogen) on a LightCycler 2.0 detection system (Roche Diagnostics, Meylan, France). Relative mRNA levels were determined using the ΔΔCt method and β-actin as reference.

**Fluorescent phospholipid transfer activity**

PLTP activity was measured as previously described, using a commercially available fluorescence activity assay (Roar Biomedical Inc, New York, NY, USA) according to the manufacturer’s instructions. This fluorimetric assay measures the transfer (unquenching) of NBD-labeled phosphatidylcholine from donor to acceptor synthetic liposomes. Absolute values were calculated from the difference between 1-minute and 15-minute time points.

**Radioactive phospholipid transfer activity**

PLTP activity was determined by using radiolabeled phosphatidylcholine-containing liposomes (14C-PC-liposomes). Briefly, 10 μmol of egg phosphatidylcholine containing 10 nmol of 14C phosphatidylcholine (100 mCi/mmol) was dried under a stream of nitrogen and recovered in 1 ml of TBS buffer. 14C-PC-liposomes were finally obtained by dispersing phospholipids with a sonifier. Transfer of radiolabeled phospholipids was measured by incubating at 37 °C an aliquot of 5 μl of each plasma sample with 14C-PC-liposomes (125 nmol of phosphatidylcholine) and HDL₃ (250 μg of protein) in a final volume of 400 μl. In ‘4°C blank’ controls, mixtures were maintained at 4°C.
Phospholipid liposomes were subsequently precipitated by the addition of 300 µl of a 500 mmol/l NaCl, 215 mmol/l MnCl$_2$, and 445 U/ml heparin. ‘Total Count’ controls received 300 µl of TBS buffer instead of the MnCl$_2$/heparin precipitant reagent. Successively, tubes were vortexed, left for 10 min at room temperature and, finally, the precipitate was removed by a 10-min centrifugation at 10,000 rpm in an Eppendorf ultracentrifuge. PLTP activity was calculated as the rate of total radiolabeled phospholipids (‘Total Count’ control) which were transferred from liposomes to HDL$_3$ after deduction of ‘4°C blank’ control values.

**LCAT activity**
Plasma lecithin:cholesterol acyltransferase activity was determined by using a commercial fluorescent assay following the instructions provided by the manufacturer (Roar Biomedical Inc, New York, NY, USA).

**Plasma parameters**
Total cholesterol, phospholipids, and triglycerides were assayed by using commercially available enzymatic kits, *i.e.* CHOD-PAD (ABX Diagnostics, Montpellier, France), PAP 150 (BioMérieux), and Triglyceride (DiaSys, Holzheim, Germany) kits, respectively. $d < 1.006$, $1.006 < d < 1.063$, and $1.063 < d < 1.210$ fractions were isolated from fasting plasma samples by sequential ultracentrifugation. The $d < 1.006$ fraction contained the triglyceride-rich lipoproteins (mainly VLDL); the $1.006 < d < 1.063$ fraction contained mainly LDL; and the $1.063 < d < 1.210$ fraction contained HDL. Low density lipoproteins were isolated by sequential ultracentrifugation as the $1.006 < d < 1.063$ fraction before determination of their cholesterol and protein content. Apo A-I and C-Reactive-Protein levels were determined by using commercial ELISA kits following the recommendation provided by the manufacturer (Uscn Life Science, China and GenWay Biotech, San Diego, CA respectively). Plasma apolipoprotein B levels were determined by analytical SDS-PAGE with Coomassie staining as previously described.$^4$ Briefly, the $d < 1.063$ plasma fractions were separated by ultracentrifugation and submitted to electrophoresis in parallel with a human apoB standard before Coomassie staining.

**FPLC analysis**
Plasma samples (200 µl) were injected into a Superose 6 HR 10/30 column (Amersham-Biosciences) that was connected to a fast protein liquid chromatography system (FPLC) (Amersham Biosciences). Lipoproteins were eluted at a constant 0.3 ml/min flow rate with Tris-buffered saline containing 0.074% EDTA and 0.02% sodium azide. Total cholesterol and triglyceride concentrations were assayed in individual, 0.3-ml fractions.
Native Polyacrylamide Gradient Gel Electrophoresis

Total lipoproteins were separated by ultracentrifugation as the $d < 1.21$ g/ml plasma fraction with one 5.5-h, 100,000-rpm spin in a TLA100 rotor in a TLX ultracentrifuge (Beckman, Palo Alto, CA). Lipoproteins were then applied to a 15-250 g/liter polyacrylamide gradient gel (Spiragel 1.5-25.0; Spiral, Couternon, France), and electrophoresis was conducted as recommended by the manufacturer. The gels were subsequently subjected to Coomassie staining with Brilliant Blue G (Sigma, St. Louis, MO.), and the distribution profiles of LDL and HDL were obtained by analysis with a Bio-Rad GS-800670 Imaging Densitometer. The mean apparent diameters of HDL and LDL were determined by comparison with protein standards subjected to electrophoresis together with the samples as previously described.\(^5\)

Quantification of alpha-tocopherol in isolated Lipoproteins

Lipophilic compounds were extracted from lipoprotein fractions by an ethanol/hexane solution (1:3, v/v), as previously described.\(^6\) The hexane fraction was evaporated under nitrogen, and finally recovered in a methanol-acetonitrile-chloroform solution (25:60:15, v/v). \(\alpha\)-Tocopherol was assayed by high-performance liquid chromatography on a Beckman Gold system equipped with a Beckman ultrasphere ODS 3-\(\mu\)m column (4.6 mm x 5.5 cm) column that was connected to a fluorescence detector (Shimadzu RF-10AXL). Tocol (SPIRAL, Couternon, France) was added to each sample as an internal standard before the extraction.

Quantification of atherosclerosis lesions

Atherosclerotic lesion areas were determined by \textit{en-face} observations. The aortic arch and the thoracic aorta were opened longitudinally, fixed in 10% buffered formaldehyde, pinned out on a black wax surface, and stained with Oil-Red-O. The percentage of the plaque area stained by Oil-Red-O to the total vessel area was determined by colour thresholding using Photoshop\textsuperscript® software. For histological analysis, serial sections of aorta were stained with hematoxylin-eosine or immunostained with specific antibody (Dako Ram-11 antibody for macrophages).

Statistics
Student’s $t$ test or Mann-Whitney test were used to determine significant differences between groups. Chi$^2$ test was used to determine the differences in LDL size distribution between WT and HuPLTPTg rabbits. Univariate relationships between lesion area and lipoprotein parameters were analyzed by the Spearman correlation test.
Supplemental references


Legend to supplementary figures

**Figure I.** HDL size distribution in HuPLTPTg and WT rabbits fed the chow diet. (A) Total plasma lipoproteins ($d < 1.21$ g/mL fraction) were subjected to electrophoresis on native 15-250 g/liter polyacrylamide gradients gels. (B) Pooled HDL profiles were obtained by densitometric scanning.

**Figure II.** Effect of cholesterol-enriched-diet on PLTP activity. PLTP activity in the plasma of WT and HuPLTPTg rabbits was determined before and after 8 weeks of cholesterol enriched diet by a radioactive lipoprotein independent assay. * indicates a significant difference as compared to WT rabbit, $P<0.05$ Student's $t$ test.

**Figure III.** Immunohistological analysis of atherosclerotic lesion in WT and HuPLTPTg rabbits fed a cholesterol-enriched diet for 8 weeks. Immunostaining for macrophages (RAM-11) and hematoxylin-eosin staining were performed on serial section of arteries from WT and HuPLTPTg rabbits.

**Figure IV.** Correlations between the extent of atherosclerotic lesions and HDL cholesterol or Vitamine E to VLDL+LDL Cholesterol ratio in HuPLTPTg and WT rabbits. Rhô and p values were obtained by Spearman rank test.
Table I: Lipid composition of lipoprotein classes of WT and HuPLTPTg rabbits fed the regular diet

<table>
<thead>
<tr>
<th>Lipid</th>
<th>VLDL</th>
<th>IDL/LDL</th>
<th>HDL</th>
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<tr>
<td></td>
<td>WT</td>
<td>HuPLTPTg</td>
<td>WT</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>80.4 ± 3.5</td>
<td>78.4 ± 3.9</td>
<td>21.1 ± 6.1</td>
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<tr>
<td>Phospholipids</td>
<td>5.5 ± 1.6</td>
<td>5.5 ± 1.9</td>
<td>36.5 ± 5.9</td>
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<tr>
<td>Free cholesterol</td>
<td>5.1 ± 0.9</td>
<td>6.2 ± 1.8</td>
<td>16.4 ± 6.1</td>
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<td>Cholesteryl esters</td>
<td>9.0 ± 2.7</td>
<td>9.9 ± 3.4</td>
<td>26.0 ± 10.5</td>
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<td>FC to CE ratio</td>
<td>0.71 ± 0.25</td>
<td>0.71 ± 0.39</td>
<td>0.55 ± 0.25</td>
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<tr>
<td>FC to PL ratio</td>
<td>0.98 ± 0.21</td>
<td>1.31 ± 0.75</td>
<td>0.58 ± 0.37</td>
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</table>
Table II: Lipid composition of lipoprotein classes of WT and HuPLTPTg rabbit fed a cholesterol-enriched diet for 8 weeks

<table>
<thead>
<tr>
<th></th>
<th>VLDL</th>
<th>IDL/LDL</th>
<th>HDL</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>HuPLTPTg</td>
<td>WT</td>
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<tr>
<td>% of total in lipoprotein classes</td>
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<td></td>
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<tr>
<td>Triglycerides</td>
<td>19.6±5.4</td>
<td>17.0±6.5</td>
<td>3.6±2.2</td>
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<tr>
<td>Phospholipids</td>
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<td>10.4±1.3</td>
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<tr>
<td>Free cholesterol</td>
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<td>28.7±6.3</td>
<td>16.2±1.4</td>
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<tr>
<td>Cholesteryl esters</td>
<td>43.3±8.1</td>
<td>49.6±9.6</td>
<td>69.8±3.5</td>
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<td>CL to CE ratio</td>
<td>0.76±0.21</td>
<td>0.63±0.25</td>
<td>0.23±0.03</td>
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<td>CL to PL ratio</td>
<td>7.11±3.91</td>
<td>7.94±5.25</td>
<td>1.57±0.17</td>
</tr>
</tbody>
</table>
Figure I

Panel A: Gel electrophoresis image showing the apparent diameter (nm) for HuPLTPTg and WT samples.

Panel B: Graph showing absorbance (Arbitrary units) vs. apparent diameter (nm) for HuPLTPTg and WT samples.
Figure II

Phospholipid transfer (% radioactivity)

- WT
- PLTPTg

Control

Cholesterol-enriched diet

* *
Figure III
Figure IV