Transgenic rabbits expressing human lipoprotein lipase

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Abstract

To study the functions of lipoprotein lipase (LPL) in lipid and lipoprotein metabolism and the relationship between LPL and atherosclerosis, we generated transgenic rabbits expressing the human LPL gene. A total of 4045 Japanese white rabbit embryos were microinjected with a 3.8-kb *SalI/HindIII* fragment containing the chicken β -actin promoter, human LPL cDNA and rabbit β -globin with poly (A) signals, and then transplanted into 116 recipient rabbits. Of the 166 pups born, six pups were transgenic as confirmed by Southern blot analysis. A Northern blot analysis revealed that human LPL was expressed by a number of tissues including the heart, kidney, adrenal gland and intestine. One transgenic rabbit showed up to 3-fold increased LPL activity in post-heparin plasma compared to that in nontransgenic rabbits. Human LPL expression in various tissues of transgenic rabbits was further elucidated by *in situ* hybridization and immunostaining. Since rabbits are superior to mice as a model of atherosclerosis, this transgenic rabbit model should provide a valuable tool for the study of LPL in lipid metabolism and atherosclerosis.

Introduction

Lipoprotein lipase (LPL, EC 3.1.1.34) is a glycoprotein (Mr = 60 kD) secreted by parenchymal cells of various tissues, mainly from muscles and adipose tissues (Camps et al., 1990; Eckel, 1989). The secreted rate-limiting enzyme hydrolyzes the core of the triglycerides of chylomicrons and very-low-density lipoproteins, thereby playing a pivotal role in the lipoprotein metabolism. Zilversmit (1973) first proposed that LPL plays an important role in atherosclerosis, because LPL can generate atherogenic remnant lipoproteins which may increase the cholesterol and triglyceride deposition in the arterial wall. Lindqvist et al. (1983) showed that the hydrolysis of triglyceride-rich lipoproteins by LPL is essential for the uptake of the lipoproteins by macrophages. Moreover, LPL may increase the cellular uptake of lipoprotein particles through an association with cell-

surface heparan sulfate proteoglycans (Williams and Tabas, 1995). The involvement of LPL in the pathogenesis of atherosclerosis has been further suggested by the presence of LPL in atherosclerotic lesions (Araki et al., 1998; Jonasson et al., 1987; Mattsson et al., 1993; O'Brien et al., 1992; Ylä-Herttuala et al., 1991).

To study the functions of LPL, five groups of investigators have generated transgenic mice expressing human LPL. Three groups produced transgenic mice expressing LPL in the whole body (Liu et al., 1994; Shimada et al., 1993; Zsigmond et al., 1994), while two groups produced transgenic mice with LPL expressed specifically in the muscle (Jensen et al., 1997; Levak-Frank et al., 1995). Shimada et al. demonstrated that overexpression of human LPL in transgenic mice reduced diet-induced atherosclerosis in the setting of low-density-lipoprotein (LDL) receptor deficiency, suggesting that LPL may protect against diet-

Figure 1. Southern blot analysis of rabbit genomic DNA. Genomic DNA was extracted from ears of rabbits and digested with EcoRI. The DNAs were loaded in 1% agarose gels, transferred to a Nitran nylon membrane and hybridized with a 1.58 kb ^{32}P -labeled LPL probe. Lanes A, B and C are for the founder L00-1, L00-2 and L00-3 rabbits, respectively, which died soon after birth. Lane D is for founder L01, which was used for the subsequent study.

induced atherosclerosis (Shimada et al., 1996). However, mice have a major drawback in that the lipoprotein profile of mice is different from that of humans. For example, mice lack cholesteryl ester transfer protein (CETP) and have hepatic lipase that circulates in the plasma. Unlike mice, in which high-density-lipoprotein (HDL) is the major carrier for cholesterol, rabbits are LDL-rich mammals, as are humans, and they have high levels of CETP. In addition, rabbits are sensitive to a cholesterol diet and develop atherosclerosis within a short period. For these reasons, several transgenic rabbit models have been produced, including human hepatic lipase (Fan et al., 1994), apolipoprotein (apo) B-100 (Fan et al., 1995), apoB mRNA editing protein (Yamanaka et al., 1995), apoE (Fan et al., 1998), lecithin-cholesterol acyl transferase (Hoeg et al., 1996) and apoA-I (Duverger et al., 1996) transgenic rabbits. In the present study, we attempted to generate transgenic rabbits expressing human LPL as an alternative model for the investigations of LPL in atherosclerosis and lipoprotein metabolism. We report here, for the first time, the initial characterization of human LPL transgenic rabbits.

Materials and methods

Generation of human LPL transgenic rabbits

Transgenic rabbits were produced by the method described previously (Fan et al., 1994; Hammer et al., 1985; Taylor and Fan, 1997). In this study, specific pathogen-free Japanese white rabbits (Tokyo Laboratory Animals Co., Tokyo, Japan and Japan SLC Inc., Hamamatsu, Japan) were used. Donor rabbits (4–6 months old) were superovulated by an intramuscular injection of 150 IU of pregnant mare's serum (Sigma, St. Louis, MO) on day 1. On day 4, donor rabbits were mated with fertile male rabbits and then injected with 150 IU of human chorionic gonadotropin (Sigma) intravenously. Embryos were collected from oviducts 17–19 h later at the Animal Center of University of Tsukuba. For the microinjection, we used 3.8-kb CAL-1 construct, which contains the chicken β -actin promoter (Niwa et al., 1991), the human LPL cDNA (Wion et al., 1987) from pRM01 including 117 bp of 5'-untranslated and 41 bp of 3'-untranslated regions, and rabbit β -globin with poly(A) signals. This construct has been used for transgenic mice (Shimada et al., 1993). The construct DNA for microinjection was resuspended at 4–8 ng μl^{-1} in 10 mM Tris (pH 7.5), 0.25 mM EDTA.

The microinjection to embryos was performed under an inverted differential interference contrast microscope (IX70, Olympus, Tokyo) at 200 $\times$ magnification. The injected embryos were transferred through the fimbrial end of the oviducts of a pseudopregnant foster mother (8–10 months old). After the surgery, all recipient rabbits were transported to the Animal Facility of Tsukuba Research Center of Yamanouchi Pharmaceutical Co., Ltd. (Tsukuba, Japan).

Detection of transgene

An ear biopsy specimen was obtained from rabbits to isolate genomic DNA, 2–3 weeks after their births. Tissues were digested in digestion buffer (50 mM Tris-HCl, 100 mM EDTA, 0.5% SDS containing 200 $\mu\text{g ml}^{-1}$ proteinase K) at 55 $^{\circ}\text{C}$ overnight, extracted with phenol:chloroform:isoamyl alcohol (25:24:1 vol/vol) (Gibco BRL, Life Technology, Gaithersburg, MD), and precipitated in ethanol. The existence of the transgene was detected by Southern blotting using a human LPL cDNA probe of a 1.58 kb EcoRI-cut fragment. Ten μg of genomic DNA digested with an EcoRI restricted enzyme was subjected to electrophoresis in a 1% agarose gel, and transferred to a Nitran nylon

Table 1. Analysis of LPL in normal and transgenic rabbit

	LPL protein ^a ($\mu\text{g ml}^{-1}$)		LPL activity ^b ($\mu\text{mol FFA ml}^{-1} \text{h}^{-1}$)	
	Pre-heparin	Post-heparin	Pre-heparin	Post-heparin
Normal rabbits (n = 3)	0.39 $\pm$ 0.09	0.96 $\pm$ 0.08	2.00	8.64 $\pm$ 2.42
Transgenic rabbit (n = 1)	1.48	3.95	0.52	19.80

^a LPL protein: 1.36–3.21 $\mu\text{g ml}^{-1}$ (human, post-heparin) using monoclonal antibody, detects both rabbit and human LPL.

^b LPL activity: 10.2 $\pm$ 3.0 $\mu\text{mol FFA ml}^{-1} \text{h}^{-1}$ (human, post-heparin) using ^{14}C labeled triolein phosphatidylcholine as described in Materials and Methods.

Figure 2. Northern blot analysis of human LPL mRNA tissue distribution in transgenic rabbit and in normal rabbit. Ten μg of total RNA was electrophoretically fractionated on a 1% agarose gel and transferred to a nylon membrane. The membrane was hybridized with the human LPL probe as described. The membrane was rehybridized with a human β -actin probe to confirm the amounts of RNA which had been loaded in each lane.

membrane by a Turboblotter system (Schleicher and Schunell, Keene, NH). Membranes were hybridized with the ^{32}P -labeled cDNA probe synthesized with Prime-It II random primer labeling kit (Stratagene, La Jolla, CA).

Isolation and analysis of RNA

The distribution of human LPL mRNA was examined in a Northern blot analysis. Total RNA was isolated from various tissues of the transgenic rabbit (aorta, heart, lung, kidney, adrenal, liver, spleen, intestine, stomach, muscle, thymus, brain, bone marrow, testis and epididymis) by Trizol reagent (Gibco BRL). Ten μg of RNA was denatured in the presence of dimethyl sulfoxide and glyoxal was subjected to electrophoresis in a 1.2% agarose gel and transferred to a Nitran nylon

membrane. The membrane was hybridized with the ^{32}P -labeled human LPL cDNA probe. The blot was rehybridized with a ^{32}P -labeled human β -actin probe (Clontech Laboratories, Palo Alto, CA) to confirm that equal amounts of RNA were loaded.

In situ hybridization and immunohistochemical staining

The Northern blot analysis (see below) revealed that human LPL was expressed mainly in the kidney, adrenal and intestine. To identify the cellular specificity of the LPL expression, *in situ* hybridization was performed with an ^{35}S -labeled human LPL cDNA probe synthesized with a Prime-It random primer labeling kit (Challah et al., 1995). All of the specimens from tissues were embedded in Tissue-Tek[®] O.C.T.

Figure 3. Immunohistochemical staining and *in situ* hybridization of human LPL in transgenic rabbit intestine. Epithelial cells of the intestine showed immunoreactivity with human LPL (A) and abundant LPL mRNA signals at the basal area (C). Normal rabbit intestine was used as a negative control (B and D). Original magnification 66 $\times$ for A and B, 80 $\times$ for C and D.

compound which contains 10.24% polyvinyl alcohol, 4.26% polyethylene glycol, and 85.5% nonreactive ingredients (w/w) and frozen with liquid nitrogen. Frozen sections were made, fixed in 4% paraformaldehyde and stored at -20°C until use.

A section was hybridized with 0.5×10^6 cpm of the ^{35}S -labeled human LPL cDNA probe diluted with hybridization solution (50% formamide, $4\times$ SSC, 10% dextran sulfate, $250\text{ }\mu\text{g ml}^{-1}$ salmon sperm DNA, $250\text{ }\mu\text{g ml}^{-1}$ herring DNA, $400\text{ }\mu\text{g ml}^{-1}$ yeast tRNA and $0.5\times$ Denhardt's solution), for 18 h at 37°C .

The specimens were then rinsed continuously with $4\times$ SSC and 40% formamide at 45°C (15 min, twice), $4\times$ SSC at 60°C (30 min), $1\times$ SSC at room temperature (15 min, three times) and $0.5\times$ SSC at room temperature (30 min, twice) and dehydrated with a graded series of ethanol. The slides were embedded in autoradiographic emulsion NR-M2 (Konica Co., Tokyo, Japan), and then exposed for 2–4 weeks at 4°C . The sections were then developed and fixed, and counterstained with hematoxylin and eosin. For a negative control, serial sections treated with $50\text{ }\mu\text{g ml}^{-1}$ of RNase prior to hybridization with cDNA probe were used. To further confirm LPL protein in these tissues, immunohistochemical staining was performed using the specific mouse anti-human LPL monoclonal antibody (mAb) 5D2, which was kindly provided by Dr. J. Brunzell, University of Washington, Seattle.

Analysis of LPL protein and enzymatic activity

Post-heparin plasma was taken 10 min after a bolus injection of heparin at a dose of 30 units kg^{-1} body weight, and pre-heparin plasma was taken before the injection of heparin. For the determination of the LPL protein, an enzyme-linked immunosorbent assay (ELISA) was performed by using the combination of two types of anti-human LPL mAb that recognized different epitopes on the LPL. The enzymatic activity of LPL was determined using a ^{14}C -labeled triolein emulsion substrate (Bensadoun et al., 1974).

Results and discussion

In the present study, we successfully produced transgenic rabbits expressing human LPL. Totally, 4045 Japanese White rabbit embryos were microinjected with a 3.8-kb *SalI/HindIII* fragment of DNA directed by a chicken β -actin promoter. Of the 166 pups born, six pups were shown to be transgenic by Southern blot analysis (Figure 1). The total positive rate for transgenic rabbits among the pups screened was 3.6%, reflecting the difficulty in generating transgenic rabbits compared to mice. The low efficiency for the transgenic rabbit production may be due to several factors. All rabbits (donors and recipients) were supplied by breeders and transported to our university. This may cause the recipient rabbits to suffer from physical stress leading to a lower pregnancy rate. Frequent physical checks by palpation (especially of implanted recipients) may also exert an adverse effect, resulting in abortion, absorption and cannibalism, as

we found in this study. Other optimal factors, such as the DNA concentration and buffer condition for pronuclear microinjection, should be investigated in the future.

Three of the transgenic founders died soon after birth due to poor mother's care, therefore, only three alive transgenic founders were obtained (Figure 1). In the present study, we made an analysis exclusively with founder L01. The other founder rabbits are currently being bred. The founder rabbit L01 was a male but sterile due to 'spray-leg' (Lindsey and Fox, 1994). As shown in Table 1, the human LPL level and enzymatic activity of rabbit L01 were equivalent to post-heparin LPL levels of humans ($1.36\text{--}3.21\text{ }\mu\text{g ml}^{-1}$ for protein mass and $10\pm 3.0\text{ }\mu\text{M FFA ml}^{-1}\text{ h}^{-1}$ for activity). Seventy percent of human LPL was present in the post-heparin plasma of rabbit L01, suggesting that human LPL can avidly bind to extracellular heparan sulfate proteoglycan on the endothelial cell surface, as does rabbit LPL. The transgenic rabbit had 3-fold higher enzymatic activity of LPL in post-heparin plasma compared to that of nontransgenic rabbits.

The Northern blot analysis (Figure 2) revealed that human LPL was expressed in multiple tissues, mainly in the kidney, adrenal and intestine. This tissue distribution of human LPL expression was similar to that of the transgenic mice produced by the same construct (Shimada et al., 1993). To identify the cellular components for human LPL expression, we performed an *in situ* hybridization and immunohistochemical staining. The intestine was used as a representative tissue for this purpose. Figure 3 shows that both human LPL protein and mRNA were present in epithelial cells of the intestine from the transgenic rabbit. Taken together, the results of our analysis of transgenic rabbits showed that human LPL was expressed in multiple tissues with enzymatic activity. We have also obtained LPL transgenic rabbits using macrophage-specific promoter, and the characterization of these rabbits is currently underway in our laboratory. The availability of different lines of transgenic rabbits in our laboratory should enable us to study the effects of LPL overexpression on lipoprotein metabolism and atherosclerosis.

In conclusion, we have successfully produced transgenic rabbits expressing human LPL. Since rabbits are superior to mice in terms of lipid and atherosclerosis studies, this model should provide a valuable tool for the study of the functions of LPL in lipid metabolism and atherosclerosis.

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