Transgenic Rabbits Expressing Human Apolipoprotein (a)

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Elevated plasma levels of lipoprotein (a) \([Lp(a)]\) constitutes an independent risk factor for coronary heart disease, stroke, and restenosis. Over the past years, our understanding of the genetics, metabolism and pathophysiology of Lp(a) have increased considerably. However, the precise mechanism(s) by which this atherogenic lipoprotein mediates the development of atherosclerosis remains unclear. This is partly due to the lack of appropriate animal models since apolipoprotein (a) \([apo(a)]\), a distinct component of Lp(a) is found only in primates and humans. Development of transgenic mice expressing human apo(a) has provided an alternative means to investigate many aspects of Lp(a). However, human apo(a) in transgenic mice can not bind to murine apoB to form Lp(a) particles. In this aspect, we generated transgenic rabbits expressing human apo(a). In the plasma of transgenic rabbits, unlike the plasma of transgenic mice, about 80% of the apo(a) was associated with rabbit apo-B and was contained in the fractions with density 1.02-1.10 g/ml, indicating the formation of Lp(a). Our study suggests that transgenic rabbits expressing human apo(a) exhibit efficient assembly of Lp(a) and can be used as an animal model for the study of human Lp(a). J Atheroscler Thromb, 2000; 7: 8-13.

Key words: Transgenic rabbit, Lipoprotein (a), Apolipoprotein (a), Lipoprotein metabolism

Introduction

Lipoprotein (a) \([Lp(a)]\) is an independent risk factor for the development of atherosclerosis. In many human studies, elevated levels of plasma Lp(a) have been found to be associated with an increased risk of atherosclerotic coronary heart disease, stroke and restenosis (1-3), although several studies have not been able to detect this association (4-6). The involvement of Lp(a) in the pathogenesis of atherosclerosis has been strongly suggested by the presence of Lp(a) in human atherosclerotic lesions (7-9) as well as the development of atherosclerosis in transgenic mice expressing human apolipoprotein (a) \([apo(a)]\) after receiving a high-fat diet (10-13).

The Lp(a) particle closely resembles low-density lipoprotein (LDL) in both lipid composition and the presence of apolipoprotein B-100 \([apoB \cdot 100]\). Lp(a) is distinguished from LDL by the presence of an additional protein component designated apolipoprotein (a) \([apo(a)]\), which is complexed to apoB-100 by disulfide linkage (1). Partial amino acid sequencing followed by complete analysis of the cloned apo(a) cDNA sequence revealed extensive homology between apo(a) and plasminogen (14). Plasminogen, like several other proteases of the blood clotting system, contains several cysteine-rich sequences called kringle domains. Apo(a) contains 12-51 copies of one particular kringle, most similar to plasminogen kringle 4 (K4), followed by a single copy of a kringle 5-like sequence and a protease-like domain. This has led investigators to suggest that the pro-atherogenic association of high Lp(a) concentrations may result from apo(a)’s sequence and structural similarity to plasminogen, leading to an inhibition of the anti-thrombotic effects and anti-proliferative effects of plasminogen on smooth muscle cells (15, 16).

Despite the progress in the clarification of the Lp(a) phenotype and genotype, the functions of Lp(a) have not been fully elucidated in vivo. This is mainly due to the lack of appropriate experimental animals since apo(a) is naturally present exclusively in Old World monkeys and humans, while one nonprimate species, the hedgehog, has independently evolved an apo(a)-like protein (17, 18).
The development of transgenic mice expressing either human apo(a) cDNA (19) or genomic DNA (20, 21) provided a means of studying apo(a) functions. More recently, transgenic rats have also been generated using the same human apo(a) cDNA construct (22). However, unlike humans in which nearly all plasma apo(a) is associated with apoB-100 through disulfide linkage, the human apo(a) in the transgenic mice and rats circulates in a free form in the plasma rather than in association with the murine LDL (19, 22). The assembly of human Lp(a) in mice can be achieved through the double expression of human apo(a) and human apoB (23, 24), suggesting that murine apoB lacks the structural requirements necessary for Lp(a) assembly. An in vitro study by Trieu and McConathy (25) showed that among the apoBs from several mammals, only human and rabbit apoB can form a disulfide bond with recombinant human apo(a), to form Lp(a) particles. This notion is further supported by the finding that expression of human apo(a) cDNA by adenovirus vector in rabbits led to the formation of Lp(a)-like particles in plasma (26). If this hypothesis is true, it is likely that transgenic rabbits expressing human apo(a) may provide an improved Lp(a) animal model. In addition, rabbits have been widely used for investigations of atherosclerosis and lipoprotein metabolism (27-29). To create a new relatively large-animal model for the study of the biological functions of Lp(a), we (30) and others (31) have developed a transgenic rabbit model that expresses human apo(a) in plasma. This article reviews recent results obtained from transgenic rabbits expressing human apo(a) (30).

Generation of Human Apo(a) Transgenic Rabbits

Transgenic rabbits were generated according to the method described previously (27-29). The details for the method of transgenic rabbit production have been reviewed in a recent publication (32). In the present study, specific-pathogen-free Japanese White rabbits (Tokyo Laboratory Animals Co., Tsukuba, Japan) were used. For the pronuclear microinjection, we used two human apo(a) transgenic constructs consisting of the full-length human apo(a) cDNA fragment containing 17 copies of kringle 4 repeats under the control of either the mouse transferrin promoter (TfHa17) as shown in Fig. 1, or the human apoAl promoter (AlHa17) (13).

After three years’ efforts, we generated three lines of transgenic rabbits. Table 1 summarizes the results of our attempts to develop human apo(a) transgenic rabbits. Among 96 pups obtained, 11 pups were transgenic as determined by Southern blot analysis of genomic DNA extracted from ear biopsy specimens. Three transgenic rabbits died before weaning due to poor maternal care. Autopsy of these dead pups did not reveal any morphological abnormalities. Eventually, eight live transgenic founders were obtained. Western blot analysis using anti-human apo(a) monoclonal antibody (a gift from Prof. Hamaguchi, H.) revealed that three transgenic rabbit founders with the TfHa17 transgene expressed human apo(a) in their plasma, whereas the other 5 founders (2 rabbits with TfHa17 and 3 rabbits with AlHa17 transgene) did not have detectable apo(a) in the plasma. The reasons for non-expression in transgenic founders are currently unknown, but we speculate that this is probably due to positional effects on the transgene, which is commonly found in non-expressive transgenic animals. The A01 transgenic founder was mated with nontransgenic rabbits to establish a line. The successful germline transmission of the transgene was verified by Southern blot analysis (Fig. 2A). Northern blot analysis showed that the human apo(a) mRNA was expressed mainly in the liver and kidney in transgenic rabbits (Fig. 2B). Hepatic expression of human apo(a) was further confirmed by immunohistochemical staining and in situ hybridization.

Assembly of Lp(a) in Transgenic Rabbit Expressing Human Apo(a)

The plasma level of human apo(a) was determined by an enzyme-linked immunosorbent assay (ELISA) using human recombinant apo(a) as a standard, which was performed in Dr. Marcovina’s laboratory, University of Seattle. The antihuman apo(a) monoclonal antibody

Table 1. Summary of human apo(a) transgenic rabbits

<table>
<thead>
<tr>
<th>Embryos Transferred</th>
<th>Recipients</th>
<th>Pups Born</th>
<th>Transgenic</th>
</tr>
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<tbody>
<tr>
<td>2,838</td>
<td>86</td>
<td>96</td>
<td>11*</td>
</tr>
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Transgene expression was confirmed by Southern blot analysis with human apo(a) cDNA probe.

*Eleven transgenic rabbits were identified and seven were alive. Among them, only three transgenic rabbits showed expression of human apo(a) protein in the plasma.

Fig. 1. Human apo(a) construct for transgenic rabbits. The hepatic expression vector (TfHa17) contains the intron, and the transcription termination signal from SV40. Human apo(a) cDNA contains 17 kringle 4 domains. This construct is provided by Dr. Lawn, RM (Stanford University) (see reference 19).
A. Southern blot analysis

B. Northern blot analysis

Fig. 2. Southern and Northern blot analysis of transgenic rabbits. Panel A. Southern blot analysis of rabbit genomic DNA. Genomic DNA was extracted from the ears of F1 (lane 1 and 2) and F2 (lane 3) rabbits (A01 line). Panel B. Northern blot analysis of apo(a) mRNA tissue distribution in transgenic rabbits. Rehybridization of the membrane with a human β-actin probe showed that similar amounts of RNA had been loaded in each lane. (Reproduced with permission from Biochem Biophys Res Commun, 255: 639-644, 1999.)

(mAb) a-40 used for ELISA recognized a unique epitope located in kringle type 9 and had the same affinity for free apo(a) and apo(a) linked to apoB (33). Average plasma human apo(a) levels in transgenic rabbits were 11 nM, which was similar to transgenic mice (13) and equivalent to 3 mg/dl of Lp(a) based on Lp(a) particle MW ~3,000,000 (Richard M. Lawn, written personal communication).

Agarose gel analysis shows that human apo(a) in the transgenic rabbit plasma was located in the pre-β position, with a similar mobility to that of human plasma apo(a). Basically, this level of expression of human apo(a) in the transgenic rabbits did not result in any changes in the lipoprotein profile compared to that of their nontransgenic littermates, suggesting that low levels of Lp(a) (3 mg/dl) in transgenic rabbits do not play an important role in cholesterol transport in the plasma. There was no difference in apo(a) concentration between the males and females at the age of 3-6 months. After serial breeding, one homozygous male rabbit was obtained (Fig. 2A). This line of transgenic rabbits were used for the following study.

To assess apo(a) and apoB interactions, plasma isolated from transgenic rabbits was adjusted to a density of 1.215 g/ml with potassium bromide and then subjected to ultracentrifugation as described by others (19). Aliquots of total plasma and the top (d < 1.215 g/ml) and bottom (d > 1.215 g/ml) ultracentrifugation fractions after dialysis were electrophoresed on a 3.5% nondenaturating polyacrylamide gel without sodium dodecyl sulfate (SDS) (19). This method was shown previously to separate free apo(a) from lipoprotein-bound apo(a). In human plasma, nearly all apo(a) was found in the top ultracentrifugation fraction (i.e., associated with lipoproteins) (Fig. 3A). We found that there was an efficient assembly of Lp(a)-like particles in transgenic rabbits. About 80% of the apo(a) in the transgenic rabbits was present in the density fractions <1.215 g/ml in the plasma as shown in the nondenaturing polyacrylamide gel analysis (Fig. 3A) and the apo(a)/apoB complex exhibited the same molecular mass range, estimated by gel electrophoresis as human Lp(a). The rest of the apo(a) was present as a free form not associated with rabbit apoB, suggesting that a certain fraction of apo(a) was either noncovalently bound or was unavailable for linkage in vivo, or became dissociated after sample collection. The efficient assembly of Lp(a) particles in transgenic rabbits is consistent with a recent report by Rouy et al. (31).

Like Lp(a) isolated from human plasma, the transgenic rabbit apo(a)/apoB complexes were sensitive to reducing agent, suggesting that human apo(a) and rabbit apoB were held together by a disulfide bond (Fig. 3B). Under nonreducing conditions, the apo(a)/apoB complex in transgenic rabbit plasma existed as a much larger molecular weight form similar to human Lp(a), while free apo(a) was present only in the lipoprotein-free fraction (bottom) as a smaller molecular weight form. Upon reduction (with β-mercaptoethanol), two apo(a) bands were visible in both the top and bottom fractions: a larger protein (~500 kDa) similar in size to the rabbit apoB, and a smaller protein (~300 kDa) (Fig. 3B). Two bands of apo(a) have also been reported in transgenic mice (19) and transfected cells using the same apo(a) cDNA construct (34). The presence of the smaller apo(a) band in transgenic rabbit plasma is unexpected based on the size of the apo(a) cDNA used in the current study. It may represent a precursor of the mature, 500 kDa form (34) or may result from a recombinational event during the process of inte-
gration of the transgene into rabbit embryo genome. Both big- and small-sized apo(a); however, can bind to rabbit apoB.

Lipoprotein density fraction analysis (Fig. 4) showed that apo(a) in transgenic rabbit plasma had a density range from \(d = 1.02\) to \(1.10\) g/ml, which is similar to that in human plasma. Lp(a)-like particle formation in transgenic rabbit plasma was further indicated by the colocalization of apo(a) with apoB at the pre-\(\beta\) position (Fig. 4). A two-step model for Lp(a) formation has been proposed in which the Type 6 or 7 kringle-4-like domain of apo(a) is involved in the initial noncovalent interaction between apo(a) and apoB (Cys-3304-3317) (35). Two studies using site-specific mutagenesis of human apoB transgenes in mice have shown that a single cysteine in human apoB (Cys-4326) is required for covalent interaction between human apoB and apo(a) (36, 37). However, a recent study showed that in rabbit apo-B, the cysteine at the position homologous to the site of apo(a) attachment in human apoB is replaced by a tyrosine residue (31); therefore, the site of the cysteine residue in the rabbit apoB molecule involved in covalent interaction with apo(a) awaits molecular cloning of the full-length rabbit apoB gene.

**Fig. 3.** Immunoblotting analysis of transgenic rabbit plasma apo(a). Aliquots of plasma and the top (\(d < 1.215\) g/ml) and the bottom (\(d > 1.215\) d/ml) ultracentrifugation fractions were separated by either 3.5% non-denaturing polyacrylamide gel electrophoresis (Panel A) or 3.5% SDS-PAGE (Panel B) under nonreducing (left) or reducing (right) conditions. Human plasma was used as a control. To indicate the molecular weight, rabbit apoB is shown on the right. (Reproduced with permission from Biochem Biophys Res Commun, 255: 639-644, 1999.)

**Fig. 4.** Apo(a) distribution in transgenic rabbit plasma. The plasma lipoproteins from a fasting nontransgenic male rabbit (top of each panel) and a transgenic male rabbit (bottom of each panel) were isolated by sequential density ultracentrifugation. Lipoproteins (8 µl) were visualized with Fat red 7B, which stains neutral lipids. To analyze the distribution of apo(a) and apoB, 2 µl of each lipoprotein fraction was resolved by 1% agarose gel electrophoresis. (Reproduced with permission from Biochem Biophys Res Commun, 255: 639-644, 1999.)
In addition to providing important insights into Lp(a) assembly and apo(a) expression, several unique features of transgenic rabbits make these animals a good model for the study of Lp(a) and atherosclerosis (see review by Fan et al. (32)). Unlike mice, in which high density lipoprotein is the major carrier of cholesterol, rabbits are rich in LDL-rich mammals, like humans, and have substantial levels of cholesteryl ester transfer protein. One drawback of our current transgenic rabbits is that the Lp(a) plasma levels in the rabbits (~3 mg/dl) are not as high as that levels in humans at risk for atherosclerosis. In humans, Lp(a) levels that exceed 30 mg/dl are considered atherogenic. Therefore, we are trying to generate transgenic rabbits expressing high levels of human apo(a) in their plasma using different promoters. However, Lp(a) is not normally present in rabbits and its significance (~3 mg/dl) in atherosclerosis is unknown. In a preliminary study, we found that A01 heterozygous transgenic rabbits developed enhanced atherosclerotic lesions on a cholesterol-rich diet compared to their littermates (36). Immunohistochemical study revealed that apo(a) and apoB are colocalized in atherosclerotic lesions in the aorta and coronary arteries of transgenic rabbits fed a cholesterol-rich diet for 16 weeks. This study is currently being extended. Furthermore, efforts are now being directed toward developing Lp(a) transgenic rabbits expressing higher levels of apo(a) in order to evaluate the effects on development of atherosclerosis.

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