Apolipoprotein(a) Yeast Artificial Chromosome Transgenic Rabbits

Lipoprotein(a) Assembly with Human and Rabbit Apolipoprotein B*

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Didier Rouy‡, Nicolas Duverger‡, Suan D. Lin§, Florence Emmanuel‡, Louis-Marie Houdebine‡, Patrice Deneuf‡, Celine Viglietta‡, Elaine Gong§, Edward M. Rubin§, and Steven D. Hughes**

From the §Ernest Orlando Lawrence Berkeley National Laboratory, Life Sciences Division, Human Genome Center, Berkeley, California 94720; ¶Rhone-Poulenc Rorer-Genceel, Atherosclerosis Department, Centre de Recherche de Vitry-Alfortville, 94403 Vitry sur Seine Cedex, France; and the ¶Institut National de la Recherche Agronomique, Jouy-en-Josas, 78352 France

The in vivo analysis of lipoprotein(a) (Lp(a)), an independent atherosclerosis risk factor in humans, has been limited in part by its restricted distribution among mammals. Although transgenic mice have been created containing Lp(a), the relatively small size of the mouse has precluded some studies. To examine the properties of this molecule in a significantly larger mammal, we have used a 270-kilobase yeast artificial chromosome clone containing the human apolipoprotein(a) (apo(a)) gene and a 90-kilobase P1 phagemid clone containing the human apolipoprotein B (apoB) gene to create transgenic rabbits that express either or both transgenes. Expression of both transgenes was tissue specific and localized predominantly to the liver. Average apolipoprotein plasma levels in the rabbits were 2.5 mg/dl for apo(a) and 17.6 mg/dl for human apoB. In contrast to observations in apo(a) transgenic mice, we found that apo(a) plasma levels in the rabbits were stable throughout sexual maturity. Also, apo(a) formed a covalent association with the endogenous rabbit apoB albeit with a lower efficiency than its association with human apoB. The analysis of Lp(a) transgenic rabbits has provided new insights into apo(a) expression and Lp(a) assembly. In addition, these transgenic rabbits potentially will provide an improved experimental model for the in vivo analysis of Lp(a) and its role in promoting atherosclerosis and restenosis.

Lipoprotein(a) is a complex particle composed of a lipid moiety and two disulfide-linked subunits: apolipoprotein B-100 (apoB1-100) and apolipoprotein(a) (apo(a)). The presence of apo(a), a hydrophilic glycoprotein structurally related to plasminogen, distinguishes Lp(a) from low density lipoprotein (LDL) and confers its characteristic biological and physical properties. Although many biological properties of Lp(a) have not been resolved, its clinical importance is highlighted by numerous studies correlating elevated plasma levels of Lp(a) with increased incidence of cardiovascular disease and stroke (reviewed in Refs. 1 and 2).

Apo(a) is naturally present exclusively in old world monkeys, humans, and one nonprimate species, the European hedgehog. Such limited distribution of apo(a) among mammals has limited studies of its in vivo properties. The development of transgenic mice expressing human apo(a) cDNA provided a means to test hypotheses accounting for the effect of Lp(a) on the vasculature. Specifically, these mice have been used to examine the ability of apo(a) to promote atherogenesis by inhibition of plasmamin formation and associated consequences (3, 4). Studies of apo(a) transgenic mice have also led to several important insights into Lp(a) assembly, including the observation that apo(a) was unable to form a covalent association with LDL containing murine apoB (5). This result, coupled with evidence for Lp(a) formation when the mice were infused with human LDL or expressed a human apoB transgene (6, 7), suggested that murine apoB lacked structural requirements necessary for Lp(a) assembly. This finding was not completely unexpected in light of the sequence specific interactions between apo(a) and apoB believed to mediate assembly of Lp(a). Two studies using site-specific mutagenesis of human apoB transgenes in mice have reported localization of a single cysteine in human apoB (Cys-4326) that provides the site of attachment for apo(a) (8, 9).

The regulation of apo(a) gene expression has been studied in transgenic mice containing a human apo(a) genomic clone (10). This transgene comprised the apo(a) gene along with its native promoter and cis-acting elements present within the approximately 60 kilobases of 5'- and 80 kilobases of 3'-flanking DNA. The transgene was more efficiently expressed (i.e. all of the apo(a) transgenic founder lines created containing an intact transgene expressed apo(a)) and resulted in significantly higher plasma apo(a) levels than observed in mice containing an apo(a) cDNA construct. A surprising finding in mice expressing the human apo(a) genomic transgene was the profound sex hormone-induced changes in apo(a) expression, far surpassing the magnitude of androgen- and estrogen-related changes observed in humans. These changes were, however, qualitatively similar in humans and transgenic mice. In both cases, these hormones lower apo(a) plasma levels.

Transgenic rabbits represent an increasingly utilized approach to the study of apolipoproteins and their impact on lipoprotein metabolism and atherogenesis (11, 12). The advantages of this animal, compared with the mouse, relate in part to its relatively larger size, enabling facile studies of vascular injury and restenosis. In addition, whereas rabbits are similar to mice in lacking apo(a) and Lp(a), their lipoprotein profile more closely mimics that of humans with LDL as the predom-
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Fig. 1. PCR analysis of human apoB and apo(a) in transgenic rabbits. Genomic DNA prepared from founder animals and progeny derived from crossing apo(a) and apoB transgensics were subjected to PCR as described under "Materials and Methods." The apo(a) transgenic founder (sample 2) contained the complete apo(a) genomic clone as judged by amplification with four different primer sets: plasminogen 5′ region (PMG 5′), apo(a) 5′, apo(a) 3′, and apo(a)-like gene 3′ (positions indicated below diagram of the genomic clone). ApoB transgenic founders (sample 1) were detected using a single PCR reaction specific for human sequences. Analyses of representative progeny from the apo(a)/apoB matings, shown in samples 3 and 4 (apo(a) only and combined apo(a)/apoB, respectively), confirmed transgene transmission. For each primer set, apo(a) or apoB genomic clone DNA was included as a positive control (sample 5).

RESULTS

DNA Preparation and Characterization—The YAC clone containing the human apo(a) gene (clone 366H2 in the Centre d’Etudes du Poly- morphisme Humain library) has previously been described (10). The YAC was separated from the yeast chromosomes by pulse field gel electrophoresis. The band containing the YAC was excised from Sea- Plaque GTG-agarose (FMC, Rockland, ME), treated with beta-agarase (New England Biolabs, Beverly, MA), and then dialyzed against 0.1 M NaCl, 10 mM Tris, 0.1 mM EDTA (14). The 90-kilobase P1 phagemid containing the human apo(a) gene has been previously described (7).

Production and Screening of Transgenic Rabbits—Production of transgenic rabbits utilized methods previously described by Duverger et al. (12). Briefly, New Zealand White adult female rabbits were super- ovulated and then mated on the same day that luteinizing hormone was injected. Embryos were collected 17 h later. Embryo injection, transfer to pseudopregnant females, and animal husbandry was as previously reported (11, 12). Approximately 2 pl of 1 ng/µl DNA solution containing either the human apo(a) YAC or the human apoB P1 phagemid was injected into the male pronuclei.

Rabbit DNA extracted from ear biopsies was screened for the presence of human apo(a) by polymerase chain reaction (PCR) using meth- ods and primers described previously (10). Human apoB-specific primers from the promoter region of the gene were used to screen rabbits for human apoB: 5′-AGAAGGTTCCAGATGTCATAGGG and 5′-TCCA- AGTACTGCTTCAAGAACCC.

Analysis of Tissue Distribution by RT-PCR—Total RNA was ex- tracted from control and transgenic rabbit tissues using RNAstat-60 (Tel-Test B, Friendswood, TX) subjected to DNase treatment and then reverse transcribed (5 µg of RNA incubated with 10 ng of apoa1RT primer: 5′-GATGACCAAGCTTGGCAGGTTCTTCC-3′, or apoB M46 primer: 5′-CCACATTTTGTATCAGITTGTAGTACTT-3′) in a vol- ume of 12 µl at 65°C for 10 min and then supplemented with Super- script II buffer (Life Technologies, Inc.) and incubated for 1 h at 42°C. The first strand cDNA was then treated with RNase and extracted with phenol/chloroform. 5 µl of the RT product was subjected to PCR under standard conditions for Taq polymerase (Boehringer Mannheim) using 50 pmol of each primer and thermocycling as follows: (94 °C for 30 s, 62°C for 30 s, followed by 72°C for 90 s/25 cycles). Apo(a) cDNA was detected using primers (aKr1F: 5′-ACCTTGGACAAAGGCATGCTG-3′ and aKr2R: 5′-AGTACTCCACATCGACAGCG-3′). ApoB cDNA was detected using human- and rabbit- specific PCR primers (M49/M50 hu- man or M49/M50 rabbit) (15). PCR products were analyzed on 1.5% agarose gels. To demonstrate the absence of genomic DNA contamination, RNA preparations were directly subjected to the same PCR con- ditions, and no product was amplified.

Apolipoprotein and Lipoprotein Analysis—Lp(a) and human apoB concentrations were measured in rabbit plasma by a sandwich enzyme- linked immunosorbent assay. The Lp(a) assay has been described pre- viously (7) and used purified IgG prepared from goat polyclonal anti- serum to human apolipoprotein(a) (International Immunology Corp., Murrieta, CA) as both the capture and detection antibody. The assay for human apoB (B48 + B100) employed a mouse anti-human apoB anti- body (Genzyme, Cambridge, MA). Standard curves were calculated with appropriate blanks and controls using Logit-Log data transforma- tion. All reported concentrations were calculated from triplicate analysis.

To assess apo(a) and apoB interactions, plasma isolated from the transgenic rabbits was separated by SDS-polyacrylamide gel electro- phoresis on 4% polyacrylamide gels according to Laemmli (16), and proteins were transferred to a Millipore nitrocellulose membrane by immunoblotting according to Towbin (17). Where indicated, samples were reduced by the addition of denaturing sample buffer containing 2.5% 2-mercaptoethanol and incubated at 100°C for 5 min. Apo(a) was detected with goat anti-apo(a) (Valbiotech, Paris) followed by horserad- ish peroxidase-conjugated rabbit anti-goat (Bio-Rad). Human apoB was detected using horseradish peroxidase-conjugated polyclonal rabbit an- tihuman apoB (kindly provided by C. Fievet, Institut Pasteur de Lille). Protein bands were revealed by an enhanced chemiluminescence kit (Amersham) and scanned using a Hoefer GS transmittance scanning densitometer. The area of the main peaks were used to evaluate the relative proportion of free and apoB-bound apo(a) (18). The apparent molecular weights were determined by the use of prestandardized proteins (Bio-Rad).

Fractionation of Plasma Lipoproteins and Cholesterol Measure- ment—Freshly isolated plasma was fractionated by gel permeation chromatography using a Superose 6 column (Pharmacia Biotech Inc.). 100 µl of plasma was injected onto the column and run under isocratic conditions with a flow rate of 40 µl/min in 0.2 M Tris, pH 8.0, 0.27 mM EDTA, 150 mM NaCl. The effluent was monitored for absorbance at 280 nm, and 50-µl fractions were collected during the run and subsequently assayed for cholesterol using a commercial chole- terol assay kit (Boehringer Mannheim). A 20-µl aliquot of cholesterol-containing fractions was used to detect the presence of apo(a) and Lp(a) using electrophoresis and immunoblotting as described above.

RESULTS

Generation of Transgenic Rabbits—To create the apo(a) transgenic rabbits, 280 microinjected embryos were transferred into pseudopregnant females, resulting in 17 live born rabbits. Two female rabbits were transgenic, and the animal with the highest apo(a) plasma level was used in subsequent studies. PCR screening was used to identify rabbits containing the transgenes and to confirm the complete integration of the apo(a) gene and its flanking regions (Fig. 1). The apo(a) founder rabbits contained an intact copy of the entire YAC genomic clone, as determined by four different PCR reactions spanning the entire length of the clone. For apoB transgensics, 780 mi-
croinjected embryos were transferred into pseudopregnant females. Of the 20 live born rabbits, two transgenics were identified using a single PCR reaction specific for human sequences in the promoter region. Offspring of the apo(a) and apoB founders were positive for the appropriate markers and demonstrated a Mendelian pattern of transmission of the two transgenes. Mating between the apo(a) and apoB transgenic founders produced rabbits of the predicted genotypes, which were used in this study.

**Tissue Specific and Developmental Expression of Apo(a) and ApoB Transgenes**—To determine the distribution of apo(a) and apoB transgene expression, mRNA preparations from several tissues (liver, testes, small intestine, kidney, brain, and lung) were subjected to RT-PCR assays as a qualitative determination of transgene expression (Fig. 2). Apo(a) mRNA was detected in the liver and the testes, whereas under more sensitive conditions (increased number of PCR cycles from 25 to 30) it was also observed in the brain. The portion of the human plasminogen gene contained on the YAC was not expressed in the transgenic rabbits (data not shown). Expression of the apo(a)-like gene was not evaluated. The liver and testes were also the major sites of expression for the human apoB transgene. A relatively weak signal (<5% of signal in liver) was detected in the small intestine. As a control for the species specificity of the apoB mRNA assay, as well as for RNA quality, the RT-PCR assay was used to detect specifically rabbit apoB mRNA. As expected, rabbit apoB mRNA was detected in all of the tissues examined.

Because of the profound decrease in apo(a) plasma levels associated with sexual maturity previously noted in mice containing the apo(a) genomic transgene, we examined the effect of sexual maturity on apo(a) plasma levels in the transgenic rabbits. Plasma samples collected from rabbits at 3, 6, and 9 months of age (rabbits are sexually mature at 6 months of age) were evaluated for changes in apo(a) levels over the course of sexual maturation in the transgenic rabbits.

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Plasma samples were taken from male and female apo(a) transgenic rabbits at 3 time points after birth: 3, 6, and 9 months. 1 μl of each sample was subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions and immunoblotting as described under “Materials and Methods” using antibodies raised against apo(a).
bound apo(a) in apo(a) transgenic rabbits suggested that the rabbit apoB/apo(a) complex was formed in vivo at a lower efficiency relative to the human apoB/apo(a) complex. Patterns of apo(a) immunoreactivity were compared in several apo(a) transgenic and apoB/apo(a) transgenic rabbits by densitometric scanning of immunoblots similar to that shown in Fig. 4A. In apoB/apo(a) transgenics, approximately 20% of the apo(a) consistently migrated as free apo(a) (n = 3), with the remaining 80% covalently bound to either human or rabbit apoB. In apo(a) transgenic rabbits, the reverse distribution was observed (80% free protein and 20% bound to rabbit apoB; n = 3). Although this was not a rigorously quantitative evaluation, the consistent differences between the two groups of transgenic rabbits containing similar total apo(a) levels suggest that apo(a) forms a disulfide linkage more efficiently with human apoB than rabbit apoB. Immunoblotting of reduced plasma samples using the same human apo(a) antisera revealed only a single band representing apo(a) in both types of transgenic rabbits (Fig. 4B). The apparent molecular mass of this apo(a) was approximately 200 kDa, corresponding to an apo(a) protein containing approximately nine kringle 4-like repeats. Samples blotted with human apoB-specific antisera (Fig. 4C) gave a single band of 550 kDa, representing human apoB-100 exclusively in the plasma of rabbits containing the human apoB transgene. Concentrations of apo(a) and apoB in the transgenic rabbits were evaluated by enzyme-linked immunosorbent assay. The average concentration of human apoB in the transgenic rabbits was 17.6 mg/dl. The average total apo(a) concentration in plasma was 2.5 mg/dl with no significant difference between the apo(a) only and human apoB/apo(a) transgenics.

To further characterize the association of apo(a) with lipoproteins in the transgenic rabbits, lipoproteins were size-fractionated by gel filtration chromatography. In contrast to density gradient centrifugation methods commonly used to separate lipoproteins from plasma, this procedure can isolate lipoprotein complexes containing proteins associated by weak noncovalent interactions. Immunoblotting of plasma fractionated in this manner showed that apo(a) was present primarily in the LDL fraction in both human apoB/apo(a) (Fig. 5A) and apo(a) transgenic rabbits (Fig. 5B). LDL fractions of combined transgenic rabbits contained two distinct high molecular weight bands corresponding to the human apoB/apo(a) complex and rabbit apoB/apo(a) complex, distinguished on the basis of the lower molecular mass of rabbit apoB (320 kDa). Covalently associated apo(a), although present in the immunoblot of total plasma, was not detected in the LDL fractions of human apoB/apo(a) transgenic rabbits. LDL fractions of apo(a) transgenic rabbits contained a broad band representing rabbit apoB/apo(a) complex accompanied by a relatively larger amount of apo(a) dissociated from LDL under electrophoresis conditions and designated as free apo(a) (Fig. 5B). This analysis confirmed the association of apo(a) with lipoproteins containing both human and rabbit apoB, although in the case of rabbit apoB, apo(a) was linked through a disulfide bond in a much lower proportion of particles.

Sequence Comparison of Putative apo(a) Binding Site in Human and Rabbit apoB—Due to the surprising ability of rabbit apoB, unlike murine apoB, to form a covalent linkage with human apo(a), we made a comparison of its amino acid sequence within a region homologous to the site of apo(a) attachment in human apoB. Since the relevant portion of rabbit apoB has not been reported, the carboxyl-terminal region of a rabbit apoB cDNA clone was sequenced. The derived protein sequence for rabbit apoB is shown in Fig. 6 aligned within a region of significant homology to four other reported apoB sequences including human. As in rodents and pigs, two mammals in which apoB has been demonstrated to be unable to form a covalent linkage with human apo(a) in vitro, rabbit apoB also lacks a cysteine at a position homologous to the site of apo(a) attachment in human apoB (Cys-4326). These results, in contrast to two previous reports suggesting that a cysteine residue at this position is required for covalent interaction with apo(a) (8, 9), indicate that cysteines elsewhere in the rabbit apoB molecule are capable of covalent interaction with apo(a).

**DISCUSSION**

The use of genomic clones in creating transgenic animals generally has several advantages over the use of cDNA con-
apo(a) was first suggested by that there was little or no human apoB protein production in not detectable in the plasma of transgenic rabbits, suggesting with human apo(a) under certain conditions (20). The current apo(a) interactions with apoB from a number of mammals that Consistent with findings of Fan reports of mice containing an identical apoB transgene (7, 19). In vivo expression in these animals. The tissue distribution of human apoB expression was somewhat different than that observed in transgenic mice. Human apoB expression detected in the intestinal of transgenic rabbits was far below that of endogenous apoB, suggesting that control elements required for expression in this tissue were not present in the apoB genomic construct. This is consistent with the conclusions of two independent reports of mice containing an identical apoB transgene (7, 19). Consistent with findings of Fan et al. (11), human apoB-48 was not detectable in the plasma of transgenic rabbits, suggesting that there was little or no human apoB protein production in the intestine.

The ability of rabbit apoB to form a covalent linkage with apo(a) was first suggested by in vitro studies examining human apo(a) interactions with apoB from a number of mammals that demonstrated that rabbit apoB may form a covalent interaction with human apo(a) under certain conditions (20). The current study substantiates this interaction under in vivo conditions and allows a comparison with the human apoB/apo(a) interaction. The relative proportions of covalently bound apo(a) in the two types of transgenic rabbits reflect a much higher efficiency of human apoB in Lp(a) assembly. As in the previous in vitro studies of apo(a) interactions with apoB from several different species, apo(a) exhibited specific noncovalent interactions with lipoproteins containing human or rabbit apoB, suggesting a highly conserved affinity between apo(a) and apoB. Such noncovalent interactions between apo(a) and apoB have been proposed to facilitate Lp(a) assembly by bringing cysteine residues in position for disulfide bond formation (21). This type of interaction may explain the presence of noncovalently bound apo(a) in the combined apoB/apo(a) transgenic rabbits. Apo(a) could persist in this noncovalently bound form due to the capacity of rabbit apoB to effectively trap some apo(a) in the initial binding step while whereas disulfide bond formation from this intermediate proceeds with much lower efficiency since covalent linkage must occur between cysteines that are not optimally positioned through noncovalent interactions.

In addition to providing unique insights into Lp(a) assembly and apo(a) expression, several features of the Lp(a) transgenic rabbits described in this work represent improvement over currently available animal models for investigating Lp(a), including a lipoprotein profile closely mimicking that of humans and the potential for vascular injury and restenosis studies. However, one important shortcoming of this model is that Lp(a) plasma levels in the rabbits are approximately 10-fold below the concentration deemed to put humans at risk for atherosclerosis. Although the low plasma level of Lp(a) is certainly a weakness of the transgenic rabbit model, its significance is unknown and is currently being evaluated through atherogenesis studies. Another aspect of the rabbit model that may be relevant to atherosclerosis studies is the formation of Lp(a)-like particles containing rabbit apoB. The properties of these particles, which constitute approximately 10% of the Lp(a) in human apoB/apo(a) transgenic rabbits, are entirely unknown. Their presence is obviously not representative of the human condition, and it creates an extra variable, the effects of which cannot be entirely determined through comparison to apo(a) transgenic rabbits. Considering these potential flaws in the model, it remains to be determined whether these transgenic rabbits will provide an important new resource to study the effects of Lp(a) on the progression of atherosclerosis and vascular disease.

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