The binding of animal low-density lipoproteins to human apolipoprotein(a)

Vuong N. TRIEU* and Walter J. McCONATHY

Departments of Medicine and Biochemistry, University of North Texas Health Science Center at Fort Worth, Fort Worth, Texas, 76107, U.S.A.

Lipoprotein(a) [Lp(a)] is a risk factor for coronary artery disease. It is composed of lipids and apolipoprotein(a) [apo(a)] linked to apolipoprotein B (apoB) by a disulphide bond between Cys-4057 of apo(a)'s kringle 36 and possibly Cys-3734 of apoB. We call this the covalent apo(a):apoB-Lp interaction, to distinguish it from the non-covalent apo(a)/Lp(a):apoB-Lp interaction, which is probably mediated by apo(a)'s kringle 33 and residues 3304–3317 of apoB. The non-covalent interaction could be the initial interaction which brings apo(a) and apoB together prior to covalent linkage and Lp(a) formation. The non-covalent apo(a)/Lp(a)-binding site on apoB is evolutionarily more ancient than

the covalent apo(a)-binding site on apoB. Both human and non-human low-density lipoproteins (LDLs) bind non-covalently to human apo(a)/Lp(a); however, only rabbit and human LDLs bind covalently to human apo(a). The non-covalent interaction between mouse LDL and human apo(a)/Lp(a) has a K_d of $(1.7 \pm 1.33) \times 10^{-7}$ M ($n = 3$). This explains the co-localization of human apo(a) and mouse apoB in the atherosclerotic lesions of human apo(a) transgenic mice and supports our hypothesis that the non-covalent interaction is a contributing factor to apo(a) atherogenicity.

INTRODUCTION

Lipoprotein(a) [Lp(a)], a risk factor for coronary artery disease [1–3], is a low-density lipoprotein (LDL)-like particle with a distinguishing glycoprotein, apolipoprotein(a) [apo(a)], covalently linked to apolipoprotein B (apoB) by a disulphide bond [4]. Apo(a) is highly homologous to plasminogen and its gene probably arose by duplication of the plasminogen gene [5]. It has many repeats of kringle 4-like domains, one kringle 5-like domain, and a domain analogous to the protease domain of plasminogen but lacking the activation site [5]. Lp(a) is assembled after the hepatic secretion of apo(a) and apoB into the plasma compartment. Lp(a) is formed when human LDLs are injected into apo(a) transgenic mice [6]; and baboon hepatocytes secrete apoB and apo(a) independently into the medium where they form Lp(a) [7]. We call this the covalent apo(a):apoB-Lp interaction, to distinguish it from the non-covalent apo(a)/Lp(a):apoB-Lp interaction, although both interactions are mediated by apo(a). The former interaction is mediated by Cys-4057 of apo(a)'s kringle 36 and possibly Cys-3734 of apoB [8–10]; and the latter interaction is probably mediated by kringle 33 of apo(a) [11] and residues 3304–3317 of apoB [12]. The apo(a)/Lp(a):apoB-Lp interaction could be the initial interaction which brings apo(a) and apoB together prior to covalent linkage and Lp(a) formation.

Apo(a)/Lp(a):apoB-Lp interaction has been demonstrated by affinity chromatography, affinity electrophoresis and ligand blotting [13]. When Lp(a) and LDL are mixed, they form an Lp(a):LDL complex with an intermediate mobility between Lp(a) and LDL on agarose gel electrophoresis [13]. Lp(a), immobilized on nitrocellulose membrane or coupled to Sepharose-6B, binds apoB-Lp [13]. The binding of Lp(a) to apoB-Lp is inhibited by an apoB peptide spanning residues 3304–3317 [12]. This peptide is part of the Lp(a)-binding site on apoB for three reasons. First, the peptide corresponds to the α_2 -antiplasmin minimum binding domain for plasminogen's kringle 4 which is highly homologous to apo(a)'s kringle 4-like domains. Secondly, the competitive nature of inhibition suggests that the peptide and apoB-Lp bind to Lp(a) by the same

mechanism at the same site. Thirdly, Lp(a) binds to the peptide immobilized on a solid support and is dissociated by inhibitors of apo(a)/Lp(a):apoB-Lp interaction, 6-aminohexanoic acid and L-proline. As the amino acid sequence of residues 3304–3317 is conserved among mammalian and avian apoBs [14], it is possible that all apoB-Lp can bind non-covalently to apo(a)/Lp(a).

We described here a simple assay for Lp(a) formation by incubating serum with CHO-pTEJha17, an apo(a)-producing cell line. This assay was used to confirm the failure of mouse apoB-Lp to form Lp(a) when exposed to recombinant apo(a) [r-apo(a)]. The lack of Lp(a) formation was not due to the lack of non-covalent binding between apo(a) and apoB-Lp. In fact, like human apoB-Lp, mouse apoB-Lp exhibited high affinity for human apo(a)/Lp(a). Consequently, mouse apoB-Lp is probably associated non-covalently with human apo(a) in the plasma compartment of apo(a) transgenic mice. This explains the co-localization of mouse apoB and human apo(a) in the atherosclerotic lesions of these animals [15].

MATERIALS AND METHODS

Materials

BSA (essentially fatty acid- and globulin-free), peroxidase substrate, antibodies to sheep IgG, and 6-aminohexanoic acid were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A.; Tween 20 from J. T. Baker Chemical Company, Phillipsburg, NJ, U.S.A.; prestained molecular mass standards from Life Technologies, Gaithersburg, MD, U.S.A.; streptavidin-biotinylated peroxidase complex and ECL reagents from Amersham Corporation, Arlington Heights, IL, U.S.A.; bacitracin from Calbiochem-Novabiochem, San Diego, CA, U.S.A.; and nitrocellulose sheets from BioTrace NT, Gelman Sciences, Ann Arbor, MI, U.S.A. All other chemicals were of reagent grade.

Lipoprotein isolation

Human plasma samples obtained from a local blood bank were collected by plasmapheresis from healthy subjects. To prevent

Abbreviations used: Lp(a), lipoprotein(a); apo(a), apolipoprotein(a); r-apo(a), recombinant apolipoprotein(a); apoB, apolipoprotein B; apoB-Lp, apoB-containing lipoprotein; LDL, low-density lipoprotein ($d = 1.006$ – 1.063 g/ml); FBS, fetal bovine serum.

* To whom correspondence should be addressed.

degradation by proteolytic enzymes, the plasma was adjusted with PPACK, leupeptin, NaN_3 and PMSF to final concentrations of $1 \mu\text{M}$, $40 \mu\text{g/ml}$, 0.1% and $0.5 \mu\text{M}$, respectively [13]. LDL_2 ($d = 1.030\text{--}1.063 \text{ g/ml}$) and Lp(a) from high-density lipoprotein (HDL_2) ($d = 1.063\text{--}1.12 \text{ g/ml}$) were isolated by sequential ultracentrifugation and column chromatography as previously described [13]. Homogeneity of Lp(a) was assessed by non-denaturing gradient gel electrophoresis (GGE) combined with immunoblotting for Lp(a) and lipoprotein B (LpB). Mouse, pig, chicken and rabbit LDL ($d = 1.030\text{--}1.063 \text{ g/l}$) were isolated from frozen sera purchased from Pel-Freeze, AK, U.S.A. The apoB of each LDL exhibited approximately the same molecular mass as human apoB, except mouse LDL , 20% of which is apoB-48. All LDL was judged to be pure by SDS/PAGE, except chicken LDL which had some contamination with HDL . Part of chicken HDL floats at a density less than 1.063 [16]; however, as HDL does not interfere with LDL binding to Lp(a) and apo(a), this was ignored. Freezing could negatively affect the apoB-Lp particles; however, frozen sera and fresh sera were tested and found to be equivalent in the Lp(a) formation assay and the apo(a)/ Lp(a) : LDL binding assay. The validity of our conclusion is supported by the concordance between our Lp(a) formation data and those reported by Van der Hoek et al. [17], and by the fact that unfrozen human LDL competes with frozen mouse LDL for binding to immobilized Lp(a) (see below).

The r-apo(a), containing 17 kringle-4-like domains and purified by lysine-Sepharose chromatography from conditioned medium of transfected human 293 cells [18], was obtained from Dr. Richard Lawn at Stanford University, Palo Alto, CA, U.S.A. Alternatively, r-apo(a) was partially purified from serum-free CHO-pTEJha17 medium. The conditioned media were passed over a lysine-Sepharose column equilibrated in PBS, pH 7.1, and the bound r-apo(a) was eluted with a solution of 0.2 M 6-aminohexanoic acid in 1.0 M NaCl. The CHO-pTEJha17 r-apo(a) was approximately 40% pure by SDS/PAGE.

Electrophoretic analyses

SDS/gradient polyacrylamide gel electrophoresis (2.5–10%), non-denaturing polyacrylamide GGE (1.5–13%), and immunoblotting were performed as previously described [13]. For apo(a) immunoblotting, alkaline phosphatase-conjugated antibody was used and blots were visualized using the phosphatase substrate from Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, U.S.A. For apoB immunoblotting, streptavidin-peroxidase conjugate was used and blots visualized using the enhanced chemiluminescent (ECL) system from Amersham, Arlington Heights, IL, U.S.A.

The r-apo(a) obtained from Dr. Lawn was used to generate sheep anti-r-apo(a). Crude antiserum was passed repeatedly over a human plasminogen-Sepharose column to remove all cross-reactivities against human plasminogen. The purified antiserum is monospecific for human apo(a); monospecificity was defined as a single arc in immunoelectrophoresis and exclusive apo(a) reactivity on immunoblotting. Ultracentrifugally isolated human LDL was used to generate sheep anti-apoB. Anti-apoB antibody was affinity-purified using an LDL -Sepharose column and biotinylated as described below for LDL . The biotinylated anti-apoB antibody is monospecific for apoB and does not react with apo(a) [13].

Ligand dot blotting

Ligand dot blotting was performed as previously described [19] using Bio-Trace nitrocellulose membrane (Gelman Sciences, Ann Arbor, MI, U.S.A.), 3,3-diaminobenzidine tetrahydrochloride

tablets (Ken En Tec, Copenhagen, Denmark) as visualizing agent, and a dot-blot apparatus (S & S, Keene, NH, U.S.A.). Intensity was determined using the Optimas system from BioScan Inc., Edmonds, WA, U.S.A. To subtract non-specific binding from total binding, background intensity was set at 0. LDL_2 was biotinylated by reacting a 1 mg/ml solution of purified lipoproteins in 0.2 M borate buffer, pH 8.5, with $60 \mu\text{l/ml}$ freshly made Biotin-X-NHS (Boehringer-Mannheim) at an initial concentration of 1.2 mg/ml in dimethyl sulphoxide [13].

Cell culture

All cell-culture reagents were obtained from Life Technologies (Gaithersburg, MD, U.S.A.). CHO-k1 was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and maintained on DMEM/F-12 (1/1, v/v) supplemented with 10% fetal bovine serum (FBS), glutamine and penicillin/streptomycin. The cells were transfected using the Transfinity calcium phosphate transfection kit (Gibco-BRL, Gaithersburg, MD, U.S.A.), selected by the antibiotic G-418 at a final concentration of 1.0 mg/ml , and maintained on serum-free medium (CHO-S-SFM) or serum-containing medium (DMEM/F-12). The expression vector pTEJ-8 was obtained from Dr. Johansen [20] and the mini-apo(a) cDNA construct was obtained from Dr. Lawn [18]. The *EcoRI* fragment carrying the mini-apo(a) cDNA was subcloned into the *EcoRI* site of pTEJ-8 to give rise to pTEJha17, an apo(a) expression plasmid.

RESULTS

Expression of recombinant apo(a)

The mini-apo(a) cDNA, encoding for an apo(a) protein with 17 kringle-4 repeats, was subcloned into pTEJ-8, giving rise to

Figure 1 Plasmid map of pTEJha17

The solid arrows indicate the neomycin-resistance gene (*Neo*), the ampicillin-resistance gene (*Amp*), and the ubiquitin promoter (*UbC* promoter). The mini-apo(a) cDNA is the *EcoRI* fragment downstream of the *UbC* promoter. The signal peptide (filled box), kringle-4-like domains (hatched and open boxes), kringle-5-like domain (V), and the protease domain (filled box) are indicated.

Figure 2 Expression of r-apo(a) in CHO-pTEJha17 cells

The cells, grown to confluency in F12/DMEM supplemented with 10% FBS, were washed with CHO-S-SFM and incubated for 3 days in the same medium. The secreted proteins were separated on a SDS/3–15%-PAGE and immunoblotted using sheep anti-r-apo(a). Lanes 1 and 3 are the control (CHO-k1 medium) and lanes 2 and 4 are the CHO-pTEJha17 medium. Sample size is 100 μ l of medium per lane. The samples were either treated (lanes 1 and 2) or not treated (lanes 3 and 4) with 2-mercaptoethanol.

pTEJha17 and placing the mini-apo(a) cDNA behind the UbC promoter. The structure of pTEJha17 is shown in Figure 1. The simian virus 40 (SV40) promoter was used to drive the neomycin (G-418) resistance gene. Transfection of pTEJha17 into CHO-k1 followed by G418 selection resulted in more than 50 clones. The transfected cells were pooled and named CHO-pTEJha17; cloning was not attempted.

An immunoblotting experiment demonstrating apo(a) expression by CHO-pTEJha17 is shown in Figure 2. On SDS/PAGE, conditioned medium from CHO-pTEJha17 exhibited an apo(a) band with a non-reduced molecular mass slightly less than apoB (approx. 400 kDa) as expected for glycosylated, full-length r-apo(a). An additional 150 kDa apo(a) band was also detected. Both exhibited a decrease in mobility following reduction by 2-mercaptoethanol due to the unfolding of their kringles and a decrease in immunoreactivity against anti-r-apo(a) raised against purified native r-apo(a). The r-apo(a) from CHO-pTEJha17 cells was partially purified by lysine-Sepharose affinity chromatography and was found to have the same binding constant (K_D) for apoB-Lp as pure r-apo(a) from Dr. Lawn, $(5.8 \pm 2.6) \times 10^{-8}$ M ($n = 3$) and $(1.0 \pm 0.4) \times 10^{-8}$ M ($n = 3$), respectively.

During extended propagation of CHO-pTEJha17, the expression level of full-length r-apo(a) dropped from 83% at 1 week in culture, to 58% after 2 months in culture, and 27% after 6 months in culture. This suggested that there are two subpopulations of apo(a)-producing cells: one producing the full-length r-apo(a) and one producing the smaller-molecular-mass r-apo(a). The former population seems to be at a competitive disadvantage and was lost gradually during culture. The latter population probably arose from an *in vivo* intramolecular recombination event which eliminated much of the coding sequence for the kringle-4 repeats.

Development of a non-radioactive assay for Lp(a) formation

Having constructed CHO-pTEJha17, we developed a simple, non-radioactive assay for Lp(a) formation. The CHO-pTEJha17 cells were incubated with medium supplemented with 10% serum; and after 24 and 48 h, the medium was removed and immunoblotted for Lp(a) following separation on a non-reducing SDS/3–15%-PAGE. As shown in Figure 3, the incubation of human serum with CHO-pTEJha17 resulted in a higher-molecular-mass apo(a) complex. Five facts show this complex to be Lp(a). First, the complex moved within the same molecular mass range as human Lp(a) on non-reducing SDS/PAGE (Figure 3). Secondly, like Lp(a), the complex reacted against both anti r-apo(a) and anti-apoB (Figure 4). However,

Figure 3 Lp(a) formation in the presence of animal sera

A confluent monolayer of CHO-pTEJha17 was incubated with F12/DMEM supplemented with 10% serum from chicken, rabbit, pig, mouse, human or fetal cow. Aliquots (20 μ l) were removed at 0 h (3), 24 h (2), and 48 h (1), electrophoresed by SDS/3–15%-PAGE and immunoblotted with anti-r-apo(a). The asterisks and Lp(a)? indicate the positions of Lp(a) derived from degradation and aggregation, respectively. The positions of Lp(a) and apo(a) are also indicated.

Figure 4 Immunodetection of apoB in the higher-molecular-mass apo(a) complex

A confluent monolayer of CHO-pTEJha17 was incubated with F-12/DMEM (lane 1) or F-12/DMEM supplemented with 10% Lp(a) negative human serum (lanes 2–5). Aliquots (40 μ l) were removed at 0 h (lanes 3 and 5) and 24 h (lanes 1, 2 and 4), electrophoresed by SDS/3–15%-PAGE and immunoblotted with anti-r-apo(a) (lanes 1, 2 and 3) or anti-apoB (lanes 4 and 5). The band corresponding to Lp(a) reacted against both anti-r-apo(a) and anti-apoB. The apoB blot was overdeveloped to bring out the Lp(a) band. Due to overloading, the apoB, on the apo(a) immunoblot, was non-specifically stained by the alkaline phosphatase colour reagent.

similar to previous analyses [21], only a small amount of apoB participated in complex formation and exhibited an increased apparent molecular mass on non-reducing SDS/PAGE (Figure 4), superimposable on the higher-molecular-mass apo(a) complex. Thirdly, complex formation is LDL-specific: Koschinsky et al. [8] reported that human r-apo(a) will only form a disulphide bond with apoB-Lp when exposed to human plasma and we have shown here that purified human LDL is necessary and sufficient to generate this apo(a) complex (Figure 5). As complex formation did not occur without human LDL, it is unlikely that it arose from non-specific and LDL-independent reactions such as self aggregation of apo(a) and/or binding of apo(a) to non-LDL component(s). Fourthly, complex formation is species specific: only sera and LDL from rabbit and human can support Lp(a) formation (Figures 3 and 5), in concordance with data published by Koschinsky and colleagues [17]. Fifthly, like Lp(a) isolated from human plasma, the rabbit and human apo(a) complexes were sensitive to reducing agent, suggesting that their apo(a) and apoB are held together by a disulphide bond (Figure 6). Similar findings were obtained using three additional human and rabbit sera.

Lp(a) formation occurred within 24 h. At 48 h, an unknown apo(a) species exhibiting a molecular mass of greater than 1000 kDa appeared; it could represent Lp(a) particles with more

Figure 6 Rabbit and human r-Lp(a) are held together by a disulphide bond

The 48 h samples shown in Figure 5 were either not treated (lanes 1–4) or treated (lanes 5–8) with 2-mercaptoethanol, electrophoresed by SDS/3–15%-PAGE and immunoblotted with anti-r-apo(a). The samples were human (1, 5), rabbit (2, 6), chicken (3, 7), and pig (4, 8). Lp(a) is indicated by (c) and apo(a) is indicated by (f). The bars indicate the 203 kDa and 105 kDa prestained molecular-mass standards.

than one apoB and/or apo(a). Fless et al. [22] has reported Lp(a) particles with two apo(a) per apoB. Additionally, a 200 kDa apo(a) band in medium supplemented with rabbit serum and a 180 kDa apo(a) band in medium supplemented with human serum were detected at 48 h; these probably arose from proteolytic degradation of Lp(a) as they are sensitive to reducing agents and disappeared following reduction (Figure 6). The 90 kDa reactivity (Figure 3) in both rabbit and chicken sera is a cross-reaction between our anti-r-apo(a) and rabbit and chicken plasminogen; and the 180 kDa reactivity in chicken serum is a cross-reaction between our anti(sheep IgG) and chicken IgG.

As LDL concentration varies among animal sera (115 mg/dl for rabbit sera, 101 mg/dl for pig sera, 31 mg/dl for fetal calf sera, 27 mg/dl for mouse sera, and 27 mg/dl for chicken sera) [16,23], we incubated the cells with the same concentration of animal LDL in serum-free medium (CHO-S-SFM), which contains 0.4 mg/ml of BSA. A result similar to whole sera was obtained (Figure 5). Recombinant-Lp(a), generated by human and rabbit LDL, exhibited the same molecular mass as those generated by human and rabbit sera. However, in the absence of Lp(a) formation, full-length r-apo(a) was barely detectable, suggesting that it is subjected to rapid proteolysis. CHO-k1 cells are probably producing a protease; and serum from human, mouse, pig and rabbit probably deactivated it, as r-apo(a) was stable in the presence of these sera.

Figure 5 Lp(a) formation in the presence of isolated animal LDL

A confluent monolayer of CHO-pTEJha17 was incubated with CHO-S-SFM (control) or CHO-S-SFM supplemented with centrifugally isolated LDL from human, chicken, pig, or rabbit at the final concentration of 9 μ g of protein/ml. Aliquots (10 μ l) at 0 h (4), 24 h (3), 48 h (2), and 72 h (1) were electrophoresed by SDS/3–15%-PAGE and immunoblotted with anti-r-apo(a). Asterisks indicate Lp(a).

Figure 7 The binding of mouse LDL to human Lp(a)

(a) The binding curves of mouse LDL and 240 ng of immobilized Lp(a) in the presence of human LDL at 0 M (○), 1.5×10^{-7} M (△), and 4.6×10^{-7} M (□). The curves were fitted to the data using the equation $\text{LDL bound} = (B_{\text{max}} \times [\text{LDL}]) / (K_D + [\text{LDL}])$ with the GraphPad computer program. (b) The apparent K_D s of A were plotted versus the concentrations of human LDL used. The x-intercept equals $-K_i$.

Simple incubation of partially purified r-apo(a) with LDL did not result in Lp(a) formation. Neither did incubation of LDL or whole serum with conditioned medium from r-apo(a)-secreting cells result in Lp(a) formation. This is consistent with the fact that purified LDL and r-apo(a) were unable to form Lp(a) even after a week of co-incubation [24]. These data suggested that an additional factor is required for Lp(a) formation. In this regard, we found that bacitracin, an inhibitor of protein disulphide isomerase [25], is an effective inhibitor of Lp(a) formation, while having no effect on cell viability and accumulation of r-apo(a) in the medium. By pooling data from three separate experiments, we found that inhibition exhibited an IC_{50} of $19 \mu\text{M}$. This suggested that protein disulphide isomerase is essential for Lp(a) formation. Protein disulphide isomerase is a resident of the endoplasmic reticulum and plasma membrane of CHO-k1 cells [25]. Plasma membrane-associated protein disulphide isomerase probably catalyses the Lp(a) formation reaction.

Non-covalent binding of apo(a) to mouse apoB-Lp

The failure of mouse LDL to support Lp(a) formation was independent of non-covalent binding between mouse LDL and apo(a). Consistent with the evolutionary conservation of the non-covalent binding site of apoB, mouse LDL bound apo(a)/Lp(a) with a K_D of $(1.7 \pm 1.3) \times 10^{-7}$ M ($n = 3$) (Figure 7).

Table 1 Inhibition of non-covalent binding by animal LDL

The immobilized Lp(a) or r-apo(a) were reacted against 2×10^{-8} M of biotinylated human LDL in the presence of 7.5×10^{-7} M non-biotinylated LDL. The % binding was calculated as the ratio of LDL bound in the presence of non-biotinylated LDL to LDL bound in the absence of non-biotinylated LDL, multiplied by 100. The data were compiled from three separate experiments. There is no significant difference between Lp(a) and r-apo(a), except for chicken LDL ($P = 0.0326$).

Inhibitor	Binding (%)	
	Lp(a)	r-Apo(a)
Human LDL	35 ± 4.6	28 ± 5.3
Chicken LDL	14 ± 1.5^a	25 ± 6.1^b
Rabbit LDL	36 ± 5.1^c	29 ± 6.7^d
Pig LDL	54 ± 5.0^e	47 ± 10.6^f

^a $P = 0.002$; ^b $P = 0.548$; ^c $P = 0.754$; ^d $P = 0.899$; ^e $P = 0.009$; ^f $P = 0.048$.

This value, however, is significantly lower than that determined previously for the binding of human LDL to Lp(a) [$(1.05 \pm 0.21) \times 10^{-8}$ M, $n = 4$, $P = 0.0015$] [19]. Mouse LDL and human LDL are binding to the same site on apo(a)/Lp(a) because, in the presence of increasing amounts of human LDL, the apparent K_D decreased while maximum binding (B_{max}) was not affected (Figure 7). The competitive K_i was determined to be 8×10^{-8} M for human LDL which is comparable with the K_D of human LDL. Theoretically, the K_i value when human LDL is used as an inhibitor is equivalent to the K_D value when human LDL is used as ligand.

A cursory examination of other animal apoB-Lp revealed that they bind apo(a)/Lp(a). For this examination, the ability of animal LDL to competitively inhibit the binding of human LDL to immobilized apo(a)/Lp(a) was compared. As shown in Table 1, all animal LDL was able to inhibit the binding of human LDL to immobilized apo(a)/Lp(a). Rabbit LDL had the same inhibitory activity as human LDL; but pig LDL was less effective. Though chicken LDL was significantly more effective than human LDL when Lp(a) was used ($P = 0.0016$), it was not more effective when r-apo(a) was used ($P = 0.548$). There was no inhibition when the 1.23 g/ml infranate fraction from each species was tested as inhibitor.

DISCUSSION

The data indicate that only human and rabbit apoB-Lp can form a disulphide bond with recombinant human apo(a), consistent with previous results showing that mouse apoB-Lp cannot form a disulphide bond with human apo(a) [15]. Of interest are data showing that mouse LDL has non-covalent affinity for apo(a). This suggested that the lack of Lp(a) formation is probably due to the lack of a cysteine rather than the lack of non-covalent binding between apo(a) and apoB-Lp. The cysteine involved in covalent binding is located on the C-terminus of human apoB [26], which has seven cysteines (residues 2906, 3167, 3297, 3734, 3890, 4190 and 4326). Cys-2906 and Cys-3890 are present on pig apoB [27], which cannot form Lp(a) [though it can bind non-covalently to apo(a), $K_D = 2.3 \times 10^{-8}$ M (V. N. Trieu, unpublished work)]; and Cys-3167 and Cys-3297 are absent on rabbit apoB [14] which can form Lp(a). Therefore, it is unlikely that these cysteines are involved in Lp(a) formation. This leaves Cys-3734, Cys-4190, and Cys-4326 as candidates. This is consistent with data showing that Cys-3734 and Cys-4190 are free cysteines [28] and that Cys-3734 is probably the cysteine involved in Lp(a)

formation [10]. To determine which cysteine is involved in Lp(a) formation, we are attempting to obtain the amino acid sequences of the relevant apoBs. If Cys-3734 is involved in Lp(a) formation, it should be missing in chicken, mouse and cow apoBs; and it should be present in rabbit apoB. Consistent with this view, Cys-3734 is missing in pig apoB.

The fact that mouse LDL has non-covalent affinity for human apo(a) explains why mouse apoB and human apo(a) co-localized in the vascular wall of apo(a) transgenic mice. This supports our hypothesis that the non-covalent interaction is a contributing factor to apo(a) atherogenicity, i.e. Lp(a) preferentially binds to the vascular wall and promotes the binding of additional apoB-Lp. Apo(a) is probably responsible for most of apoB accumulated in the vascular wall and the ratio of three apoBs per apo(a) in the vascular wall [29] is in agreement with the reported stoichiometry of three apoBs/apo(a) in the Lp(a):apoB-Lp complex [19]. Our hypothesis can be tested using the apo(a) and/or Lp(a) transgenic mice; however, the observation that rabbit apoB, in the presence or absence of plasma proteins, will form disulphide linkage with human r-apo(a) points to the rabbit as an additional model for Lp(a) metabolism. As any gene can be delivered to the liver by either liposome-mediated gene transfer or adenoviral-mediated gene transfer, one should be able to generate rabbits transiently expressing human apo(a) to study Lp(a) metabolism and atherogenicity.

The Lp(a) formation assay described here is simple and does not involve radioactivity. It is clear, however, further improvements can be made. Most noticeable is the fact that CHO-pTEJha17 is expressing two forms of r-apo(a): the full-length r-apo(a) and a smaller truncated form of r-apo(a). To bypass this problem, we have constructed CHO-pTEJha6, which expresses a single apo(a) form with six kringle-4 repeats (kringles 33, 34, 35, 36, 37 and a fusion between kringle 1 and kringle 32). This cell line is stable and the secreted apo(a) has been shown to form Lp(a) when exposed to human serum or LDL.

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