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APOLIPOPROTEIN(a): STRUCTURE AND BIOLOGY

Angelo M. Scanu [1,2] Kei Nakajima [1] and Celina Edelstein [1]

[1] Departments of Medicine and of Biochemistry and Molecular Biology [2], University of Chicago, Chicago, Illinois 60637

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1. ABSTRACT

Apolipoprotein(a), apo(a), the distinctive glycoprotein constituent of lipoprotein(a), Lp(a), is synthesized in the liver, links covalently to apoB100-lipoprotein, and travels so linked in the plasma to tissue sites where removal mechanisms are yet undetermined. Depending on the redox status of the surrounding milieu, apo(a) may re-acquire its unbound state shown to have structural and functional properties different from those of the bound form. Apo(a) is potentially athero-thrombogenic, a property which may be influenced by its size, sequence polymorphism, type of lipoprotein it is linked to and the inflammatory state of the vessel wall. This set of variables must be taken into account when assessing the cardiovascular pathogenicity of free and bound apo(a).

2. INTRODUCTION

Apolipoprotein(a), apo(a), is a multikringle structure which in the plasma is predominantly linked by a single disulfide bridge to apoB100-containing particles referred to as lipoprotein(a), Lp(a) (1, 2). In normotriglyceridemic subjects, apo(a) is mainly bound to a low density lipoprotein, LDL (2). Because of its high prevalence, this Lp(a) species has been the one most extensively studied. However, in dyslipidemic states, apo(a) can also be linked to the apoB100 of triglyceride-rich particles, namely very low- and intermediate density lipoproteins, VLDL and IDL, respectively (1, 2). These Lp(a) species may have a physiopathological role different from that of LDL-containing Lp(a). Moreover, apo(a) can occur unbound to apoB100-containing lipoproteins, although in relatively small amounts, at least in the plasma (3, 4). This unbound apo(a) referred to as free apo(a), may be associated with small amounts of other plasma components. This article with a focus on apo(a), will provide an overview on past studies and present new perspectives based on the most recent developments in the field.

3. PROPERTIES OF APO(a)

Apo(a) is an inherited quantitative trait, the product of a high polymorphic gene (5, 6). The seminal work by McLean *et al.* (7) has shown that apo(a) consists of several kringles followed by a protease region for which no activity has been identified. These kringles have a close structural homology to those of plasminogen and on that basis, classified as KIV and KV. Of the two, and contrary to plasminogen, apo(a) KIV has 10 distinct types named from 1 to 10 (8). All of them are present as a single copy except for KIV type 2 which occurs in several repeats varying in

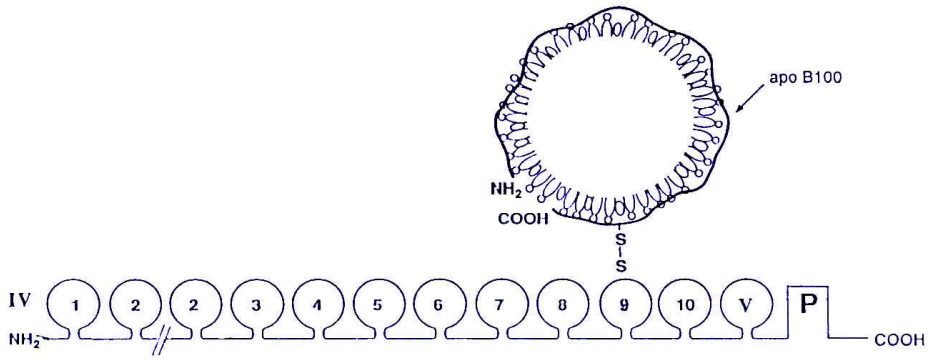

Figure 1. Schematic view of the apo(a) structure. Represented are 10 types of KIV and one KV followed by the protease domain (P). There are several KIV type 2 repeats whose number determines apo(a) size. KIV type 9 is involved in disulfide linkage with one of the unpaired cysteines in the C-terminal domain of apo B100 (see test). KIV-10 contains a high affinity lysine binding site, LBS-1, which is the only one functional in Lp(a). A second LBS, LBS-2, is masked in Lp(a) but unmasked in apo(a). This site comprises the region between KIV-6 and KIV-8. The protease domain is inactive and its function, if any, is unknown.

number within and among individuals, a polymorphism responsible for the size of apo(a) from 300 to 800 kDa (7) (figure 1). A direct documentation for this polymorphism has come from studies in which a restriction enzyme was used to cleave the apo(a) gene outside the repeat domain, generating large fragments that could be separated and identified by pulsed field gel electrophoresis followed by genomic blotting (9). This important achievement and the development of a high resolution electrophoretic technique for the separation of the apo(a) phenotypes in the plasma (10), have permitted to gain newer insights into the biology of apo(a) polymorphism although several questions still remain unresolved. Two additional polymorphisms in the apo(a) gene have been described: one is the 5' pentanucleotide repeat located in the promoter region and the other, in the coding and non-coding region that includes the C/T polymorphism at + 93 (2, 11). The effect of these two polymorphisms on the plasma levels of Lp(a) has not been clearly documented.

4. BIOSYNTHESIS

Several studies have established that the liver is the site of synthesis of apo(a) (2). Very small amounts of mRNA have been observed in the lung, brain, adrenal glands and testes of both human subjects and rhesus monkeys with no evidence, however, for protein production (5, 12). In terms of mode of synthesis the most extensive studies have been conducted in primary baboon hepatocytes showing the existence of a precursor undergoing processing to a mature form through a series of steps in the endoplasmic reticulum (N-glycosylation, folding and proteasome degradation) and the Golgi apparatus (carbohydrate addition via N- and O-glycosylation) (13). Those studies have also shown that the residence time in the ER is largely dependent on apo(a) size, the larger isoforms being retained longer than the smaller ones due to their relative sensitivity to proteasome degradation (13). This differential intracellular trafficking has been suggested to account at least in part for the inverse relationship between apo(a) size and Lp(a) concentrations in the plasma. White *et al* (14) have also related the activity of the proteasome system to apo(a) secretory rates. A shortcoming of these elegant studies is that the structure of baboon apo(a) has not been defined and, based on the results of rhesus monkey apo(a), may not be a true reflection of the human product.

5. ASSEMBLY

In vitro studies have indicated that the assembly process occurs in two steps. The first one, non covalent in nature, involving in apo(a) the region between kringles IV types 5-8 and a charged site in the C-terminal domain of apoB100. In the second step, a disulfide bridge is established between cys 4328 in located in the KIV-9 of apo(a) and cys 4057 of apoB100 (15, 16). *In vivo*, the assembly steps have not been clearly identified nor the site or sites where Lp(a) is formed. In mice transgenic for human apo(a) the intravenous injection of human LDL resulted in the rapid formation of human LP(a) suggesting that the assembly of Lp(a) can occur in the plasma (16). Studies in baboon hepatocytes (17) and HepG2 cells in culture (17) have failed to demonstrate intracellular covalent linkage between apo(a) and apoB100. In addition, White and Lanford (18) have shown that in the baboon hepatocyte the assembly may occur at the plasma membrane surface. However, the results of other investigations do not favor an exclusive extracellular mechanism. In cell lysates of primary human hepatocytes, earlier studies by this laboratory have provided evidence for the presence of apo(a)-apoB-containing lipoproteins and for their increased production when the medium was supplemented with oleate (19). Recently, a similar observation was made in HepG2 cells transfected with a human apo(a) minigene (20). Moreover, *in vivo* turnover studies using stable isotopes conducted in normolipidemic subjects, supported an intracellular mode of Lp(a) assembly by showing that the synthetic rates of apo(a) and apoB in Lp(a) were comparable but different from those of apoB in LDL (21). These discrepant findings invite additional studies with an emphasis on human subjects since currently we have no adequate animal models.

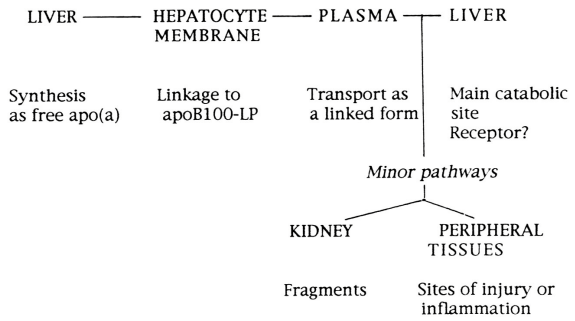

Figure 2. Main metabolic steps involving apo(a). Synthesis occurs in the liver. The predominant view is that apo(a) links to apoB100 at the surface of the hepatocyte membrane; however, other studies support an intracellular event (see text). In the plasma, apo(a) is predominantly linked to apoB100-lipoproteins, mostly LDL, a linkage which is critical for the transfer of apo(a) from the synthetic to the catabolic sites. Under normal conditions, the liver is the main site of uptake and degradation by mechanisms yet unknown. Also unknown is whether this step requires a prior modification of either bound or unbound apo(a). The kidney is involved in the excretion of apo(a) fragments whose mechanism of production remains undetermined although proteolysis has been suggested. Under normal conditions, this pathway is only minor from the quantitative standpoint. However, it may increase in magnitude in pathological situations like the nephrotic syndrome. Immunoreactive apo(a), likely representing both bound and unbound forms, may accumulate at sites of injury or inflammation and may contribute to the healing process.

6. CATABOLISM

There is still little knowledge on the mode of removal of Lp(a)/apo(a) from the circulation. Studies in rats indicated that the liver is the major site of removal of the intravenously injected human Lp(a) (22). However, whether this information applies to man remains to be established. The kidney may also play a role although likely a minor one, based on the observation that subjects with renal insufficiency show relatively high levels of plasma Lp(a) (23). The fact that LDL is one of the two constituents of Lp(a) has brought the attention of the investigators to the LDL receptor. However, its participation in Lp(a) catabolism has been ruled out particularly from the results of turnover studies in subjects with homozygous familial hypercholesterolemia (24) and the inefficiency of statins to lower plasma Lp(a) levels (2). Evidence has been presented about the role of the VLDL receptor in the internalization and degradation of Lp(a) (25). However, this receptor is only minimally expressed in the liver and thus unlikely to contribute in a major way to the clearance of Lp(a) from the circulation (26). A receptor in foam cells that binds apo(a) and some of its fragments has been reported (27); however, its physiopathological relevance has not been established. Recently, apo(a) has been shown to bind to the protein core of proteoglycans (28). Whether this type of interaction may play a role in the uptake and internalization of Lp(a)/apo(a) at the liver site remains to be established. The postulated main metabolic steps involving apo(a) are summarized in figure 2.

7. LDL – UNBOUND APO(a) IN BIOLOGICAL FLUIDS

Small amounts of LDL-unbound apo(a) have been reported in the "bottom fraction" of human plasma after ultracentrifugation (3, 4). Recently, immunoreactive apo(a) fragments have been reported (29-34). The concentrations of apo(a) have been estimated between 2 and 5% of the total plasma apo(a). However, it is likely that these estimates are inaccurate because the nature of the unbound apo(a) has not been established nor has been the proportion of fragments which are likely to differ in immunochemical properties from full-length apo(a).

How apo(a) and fragments originate from bound apo(a) is still undermined. In the case of full-length apo(a) two sources are possible: one from apo(a) that escapes interaction with apoB-containing lipoproteins and the other, from dissociation from intact Lp(a). There are no experimental data to support the first hypothesis. In terms of the second one, metabolic studies in humans have shown that from 10 to 25% of the injected Lp(a) is converted to LDL, which is then taken up by the LDL receptor (24). However, the fate of the dissociated apo(a) was not determined. This dissociated apo(a) may be the source of the fragments (32, 35) possibly by proteolytic cleavage (35, 36) at site(s) yet to be established.

8. METHODS OF ISOLATION OF FREE APO(a)

8.1. From plasma

While it is important to gain knowledge on the properties of naturally occurring free apo(a), effective and standardized procedures for its isolation are not available. The limited progress in this area may be attributed to a number of factors: relatively low abundance, undetermined proportions between full-length and fragmented apo(a), tendency of apo(a) to re-associate with apoB100 and other plasma components like fibrinogen (37), plasminogen (38), defensin (39), and probably others. Current knowledge would argue against the utilization of either ultracentrifugal or precipitation methods by themselves unless complemented by immunochemical approaches using antibodies that discriminate between free and unbound forms of apo(a). Another approach would be to exploit the knowledge derived from studies on the *in vitro* interactions between apo(a) and apoB100. To this effect, interesting are the recent studies by Herrmann *et al* (40) who isolated free apo(a) by an affinity chromatography procedure using as a ligand the segment in apoB100 between residues 3375 and 3405 representing the non-covalent binding site for apo(a). By a specific immunoassay utilizing a polyclonal anti-apo(a) antibody raised in the sheep, those authors were able to determine the concentration of free apo(a) in the plasma and found it to be increased in patients with coronary artery disease. These studies while in need of validation both from the technical and clinical standpoints, do point at the importance of including free apo(a) in the assessment of the athero-thrombogenic potential of Lp(a).

Table 1. Comparison of biological activities between bound and unbound apo(a)

Apo(a)			
Activity	***Bound***	***Unbound***	***Ref***
Binding to:			
Lysine-Sepharose*	75	90	***
Decorin, protein core**	18.4	57.8	28
Fibrinogen**	1.3	36.6	32
Fibronectin**	1.8	92.0	32

* Based on the elution with epsilon-aminocaproic acid at 22 mM for the bound and 110 mM for the unbound, apo(a) expressed as a percentage of the total sample applied, **Based on the binding avidity (Bmax) expressed in fmoles. ***C. Edelstein, unpublished observation.

8.2.From Lp(a)

The method successfully developed by Edelstein *et a*l (41) relies on the relatively higher sensitivity to reducing agents by the interchain disulfide linking apo(a) and apoB100 compared to the three intrachain disulfides present in each apo(a) kringle. The resulting free apo(a) has been shown to be stable on storage, to retain on reduced SDS gels the same electrophoretic properties as bound apo(a), to exhibit lysine binding properties and to bind to LDL, VLDL (41). This Lp(a)-derived apo(a) has also been shown to interact to members of the extracellular matrix, namely the protein core of decorin (28), fibrinogen (37) and fibronectin (42). Free apo(a) is also recognized by antibodies raised against holo Lp(a). However, in terms of the above properties, some quantitative differences exist between free and bound apo(a). The possible reasons are discussed below.

8.3. Natural apo(a) as a biological reagent

For a number of years it has been technically difficult to determine whether the biological effects of Lp(a) are due to either apo(a), its lipoprotein component or both. The availability of a recombinant apo(a) helped in this regard (43) although there are important shortcomings mainly related to its in vitro production in the embryonic kidney cells rather than in the liver, a glycosylation pattern different from that of natural apo(a) and limited access. As indicated in the previous section, an important advance in this regard has been the generation in vitro of a native form of apo(a) by subjecting Lp(a) to a mild reductive cleavage and the demonstration that this apo(a), when incubated with apoB100-containing particles, contributes to the formation of an Lp(a) indistinguishable from the naturally occurring particle (41). As a consequence of these developments, it has become possible to compare the biological action of free and bound apo(a) in the same subject. By this approach, similarities and dissimilarities between these two forms of apo(a) have been observed as summarized in table 1. These results indicate that free apo(a) is biologically more efficient than Lp(a) probably due to the unmasking of functional sites (41, 44) and the occurrence of conformational changes (45) that may follow the dissociation of apo(a) from Lp(a). A good example of the differences between free and bound apo(a) is provided by the results of the studies dealing with the lysine-mediated binding of apo(a) fibrinogen (37). In the case of Lp(a), the only functional binding site (LBS-1) is that located in kringle IV-10. On the other hand, in free apo(a), an additional functional lysine binding site, LBS-2, is present in the domain between KIV-5 and IV-8, masked in Lp(a), thus providing an additional binding arm to apo(a). The usefulness of natural apo(a) also emerged from the work dealing with a lysine-binding defective (lys-) species of Lp(a) due a Trp72 -Arg missense mutation causing LBS-1 to be inactive (46). This lys- Lp(a) is present both in rhesus monkeys (47) and in some Caucasian subjects (46). However, in each instance, the apo(a)-derived form bound to both lysine-Sepharose (41) and fibrinogen (37) indicating the unmasking of a functional LBS-2. By comparing free apo(a) and parent Lp(a) we and others have also observed that apo(a), but not LDL, mediates the binding of Lp(a) to fibrinogen (40), fibronectin (42), the protein core of decorin (28) and the endothelial cell surface (48), findings that support the importance of apo(a) by itself in the physiopathology of Lp(a).

9. CAN APO(a) BE FORMED *IN VIVO* AS A DISSOCIATION PRODUCT OF LP(a)?

Surprisingly, this is a research area which has received no or very little attention. The metabolic studies in human subjects have shown that from10 to 25% of radiolabeled Lp(a) can be converted to LDL which is then removed by the LDL receptor (24, 29). This suggests that some of the injected Lp(a) underwent a reductive cleavage causing the release of apo(a) which is then targeted to catabolic sites perhaps different from those of holo Lp(a). It will be important to establish where Lp(a) cleavage might take place. A dissociative event involving a disulfide bond would require a reductive environment unlikely to be provided by the closely buffered milieu of the circulating plasma, at least under normal conditions. A potential site could be the endothelial surface since free apo(a) binds (48), although the actual binding site has not been established. Another site could be the hepatocyte membrane which we may envisage as serving a dual role in both the assembly and disassembly of Lp(a). Evidence that apo(a) is degraded in the hepatocyte has been reported (22); however, whether such a process requires a prior dissociative event remains to be established. In approaching this issue it will be important to address separately cholesterol- and TG-rich Lp(a) species (see below) as well as physiological and pathological states. For instance, in the case of the atherosclerotic process for which an inflammatory basis has been postulated (50), inflammation may create the reductive milieu favoring apo(a) dissociation.

10. IS THE METABOLIC FATE OF APO(a) INFLUENCED BY THE LIPOPROTEIN PARTICLE IT IS ATTACHED TO?

As we already stated in the early part of this review, apo(a) circulates in the plasma mostly bound to

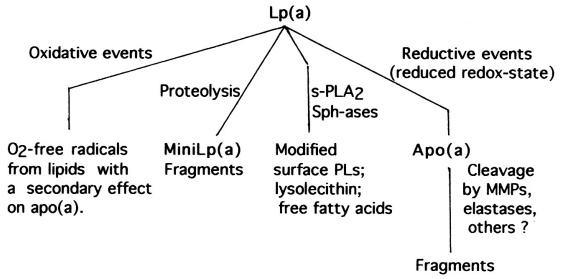

Figure 3. Potential modifications of Lp(a) in human atheroma. It is unclear whether Lp(a) undergoes modification during trans-endothelial transport. However, in the sub-endothelial intima, several events may take place in a chronology and magnitude which may depend on the inflammatory state of the surrounding tissue. The modifications may involve: 1. Lipid oxidation with a secondary effect on apo(a), 2. Proteolysis likely contributed by the action of metalloproteinases, resulting in the generation of miniLp(a) and apo(a) fragments: 2. Phospholipolysis effected by secretory phospholipase A2 and/or sphingomyelinases; 4. Reductive events causing the release of apo(a) from bound apo(a). This released apo(a) would be readily susceptible to proteolytic cleavage.

apoB100-lipoproteins. These are the particles most extensively studied and the ones which have generated the largest body of information on Lp(a). In comparison, very little is known on TG-rich Lp(a) species. Studies from this laboratory in human primary hepatocytes have provided evidence that de novo TG production relates to apo(a) synthesis (19). We have also shown that in the plasma of hypertriglyceridemic subjects, apo(a) phenotypes of a large size is preferentially associated with TG-rich Lp(a)s also known to contain apoE (51). These findings, coupled with the knowledge that plasma levels of TG-Lp(a) are low (2) have led to suggest that the TG species undergo rapid removal via receptors that recognize apoE as a ligand. ApoE may associate with TG-Lp(a)s via surface phospholipids, and according to the results of our recent studies (52), also directly to apo(a). Of note, one of the functions of apoE is to stimulate hepatic secretion of TG-rich lipoproteins. Taken together these results invite the speculation that in mixed hyperlipidemias TG-rich Lp(a)s are assembled prior to secretion.

11. IS THE LINKAGE WITH APOB100-LIPOPROTEINS CRITICAL FOR THE CARDIOVASCULAR PATHOGENECITY AND PHYSIOLOGY OF APO(a)?

A number of retrospective studies have shown an association between high plasma levels of Lp(a) and an increased incidence of atherosclerotic cardiovascular disease and have suggested that Lp(a) is an independent risk factor for this disease (2). However, discrepant views have emerged from some of the prospective studies (2). These discrepancies have been attributed to a host of factors playing a role either individually or in concert. Among them, lack of uniformity in sample storage and assay procedures, lack of agreement on the cut-off point between normal and abnormal plasma levels and differences in study design. Moreover, distinction must be made between Caucasians and African Americans since the latter have 2-3 fold higher plasma levels but not a comparable risk for cardiovascular disease (2). It has also been shown that the cardiovascular pathogenicity of Lp(a) may increase in the presence of either high plasma levels of LDL (53) low HDL (54) or both and that homocysteine may have a synergistic effect (55). All of these observations support the view that high plasma Lp(a) levels represent a dependent rather than an independent risk factor. This conclusion is in keeping with the results of epidemiological studies indicating that the cardiovascular pathogenicity of Lp(a) relating to plasma levels only applies to the middle-aged subjects (2). In this context , two separate studies have recently shown that centenarians with no apparent ischemic cardiovascular disease have either similar or higher Lp(a) levels as middle-aged control subjects (56, 57).

An additional confounding factor in assessing the intrinsic cardiovascular pathogenicity of Lp(a) is the potential that this pathogenicity may be influenced by "atherogenic" lipoproteins which apo(a) is linked to. For instance, is there a difference between an apo(a) phenotype linked to a buoyant vs. a small dense LDL or is there an "atherogenic" apo(a) phenotype irrespective of lipoprotein association? We may also ask why of the other kringle-containing proteins in the plasma, only apo(a) is linked to apoB100-containing lipoproteins, and why this linkage is always present in the animals expressing apo(a) (13). These data suggest that the formation of a disulfide bond between apo(a) and apoB100 has an important and, perhaps, essential role for the biology of apo(a). One possibility is that such a linkage insures the transport of apo(a) to tissue sites to permit its participation in the early phases of healing, a repair function which has been suggested in the past (58). This transport mechanism is also operative in the case of the artery wall. Under normal conditions, no apo(a) immunoreactive material is detected (2, 59) likely because of its inability to accumulate there due to lack of retention. In atherosclerosis, retention will occur because of the capacity of apo(a) to interact with various members of the extracellular matrix present in large amounts in the intima. Much of the retained material has been shown immunochemically to represent free apo(a) either intact or fragmented (59), an indication that two events might have occurred. One, dissociation of apo(a) from apoB, the other, degradation due to proteolytic and oxidative processes along with lipolytic events involving undissociated Lp(a) (figure 3). Thus, the attachment to apoB100 allows apo(a) to readily move in the plasma in a conformational state that prevents its association with other plasma components shown to interact with apo(a) *in vitro*: i.e. plasminogen, fibrinogen and fibronectin. Once this apoB100-mediated transport is terminated, Lp(a) reaches tissue sites, where depending on the redox status of the medium, apo(a) can re-acquire its free state and be biologically active either as a full length protein or as fragments thereof. In terms of fragments, we have earlier suggested that the ones unattached to apoB100 can recycle back into the plasma where they become targeted for excretion into the kidney (34). On the other hand, those with a high affinity for the extracellular matrix, are retained

and perhaps subsequently modified (60); and in the case of the artery wall, contribute to the progression of the atherosclerotic process (60).

12. CLOSING COMMENTS AND PERSPECTIVES

Several years after its discovery, apo(a) retains its enigmatic image with a poor understanding of its physiological function, an unparalleled genetic polymorphism, wide inter-individual variations in plasma levels, presence only in man and in some non-human primates and occurrence in the plasma predominantly in a covalent linkage with apoB100-containing lipoproteins. It is also becoming apparent that its cardiovascular pathogenicity may be modulated by the presence of other risk factors by mechanisms yet to be defined.

The absence of suitable animal models has certainly contributed to our relatively limited progress in the understanding of the mechanisms involved in the cardiovascular pathogenicity of Lp(a). Of note, while Lp(a) has been the subject of intense studies, comparatively less work has been directed at elucidating the intrinsic properties of unbound apo(a) partially due to the fact that only recently it has been possible to effect *in vitro* its safe release from Lp(a). It is now apparent that apo(a) by itself has an athero-thrombogenic potential possibly related to apo(a) size at least in the Caucasian population, and perhaps, to the type of lipoprotein species it is associated with in the plasma. Given the fact that apoB100-lipoproteins represent a family of particles differing in size, density, lipid content, composition and athero-thrombogenic potential, it should be the goal of future studies to establish whether apo(a) isoforms have a preference for given lipoprotein species. In such a case, one could have Lp(a) particles differing in cardiovascular pathogenicity depending on the nature of its constituents although apo(a) is likely be the most important determinant in this regard. Supportive of this concept are the observations both *in vitro* and *in vivo* showing that a single point mutation, Arg72 -Trp, reduces significantly the atherogenicity of apo(a) whether bound or unbound to a lipoprotein (61, 62). It is likely that additional mutations will be discovered in the future and some of them may prove to be pathogenic.

From the above, it is apparent that size, mutations and specificity of association with apoB100-lipoproteins possibly resulting in conformational changes can cause a biological variability that will need to be taken into account in future studies also in regards to apo(a) antigenicity. For instance, we cannot exclude that the antigenic reactivity of a given apo(a) phenotype may be influenced by its free or bound state and, in the latter case, whether linked to CE- or TG-rich apoB100- lipoproteins. This potential antigenic variability must be taken into account when approaching the still unresolved problem of the standardization of the immunoassays for Lp(a).

Finally there is the therapy issue. Since several studies have associated the cardiovascular pathogenicity of Lp(a) to its plasma levels, their reduction is obviously an important goal. Unfortunately, in spite of significant progress not all factors, genetic and environmental, regulating these levels have been defined. Resolving the therapeutic problem would not only be significant from the clinical standpoint but also for gaining a better understanding of the biology of free and bound apo(a). There is also a need to arrive at a clear demarcation between normal vs. abnormal plasma levels of Lp(a) and define the extent to which cardiovascular pathogenicity relates to these levels. Moreover, both Lp(a) and apo(a) can undergo post-translational modifications --- proteolytic, lipolytic and oxidative in the case of Lp(a) and proteolytic in the case of apo(a) --- varying in degree of atherogenicity. A clear understanding of the mechanisms underlying these modifications, would provide a robust basis for developing means for neutralizing potential adverse effects at the level of target organs.

To sum-up, Lp(a) with its many uncertainties continues to represent a difficult but challenging subject of investigation. Many unknowns still remain and , among them, a better understanding of the structural and biological relationships between bound and unbound apo(a). These unknowns must be resolved before effectively embarking in clinical studies which, thus far, have led to controversial outcomes. Overall, it is becoming apparent that the unveiling of the mysteries that now surround Lp(a) will require coordinated multi-disciplinary efforts.

13. ACKNOWLEDGMENTS

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Send correspondence to: Dr Angelo M. Scanu, University of Chicago, Department of Medicine, MC5041, 5841 S. Maryland Ave., Chicago, IL 60637, Tel: 773-702-1775, Fax: 773-702-4534, E-mail: ascanu@medicine.bsd.uchicago.edu