

Structural basis for the pathophysiology of lipoprotein(a) in the athero-thrombotic process

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Abstract

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Lipoprotein Lp(a) is a major and independent genetic risk factor for atherosclerosis and cardiovascular disease. The essential difference between Lp(a) and low density lipoproteins (LDL) is apolipoprotein apo(a), a glycoprotein structurally similar to plasminogen, the precursor of plasmin, the fibrinolytic enzyme. This structural homology endows Lp(a) with the capacity to bind to fibrin and to membrane proteins of endothelial cells and monocytes, and thereby to inhibit plasminogen binding and plasmin generation. The inhibition of plasmin generation and the accumulation of Lp(a) on the surface of fibrin and cell membranes favor fibrin and cholesterol deposition at sites of vascular injury. Moreover, insufficient activation of TGF- β due to low plasmin activity may result in migration and proliferation of smooth muscle cells into the vascular intima. These mechanisms may constitute the basis of the athero-thrombogenic mode of action of Lp(a). It is currently accepted that this effect of Lp(a) is linked to its concentration in plasma. An inverse relationship between Lp(a) concentration and apo(a) isoform size, which is under genetic control, has been documented. Recently, it has been shown that inhibition of plasminogen binding to fibrin by apo(a) is also inversely associated with isoform size. Specific point mutations may also affect the lysine-binding function of apo(a). These results support the existence of functional heterogeneity in apolipoprotein(a) isoforms and suggest that the predictive value of Lp(a) as a risk factor for vascular occlusive disease would depend on the relative concentration of the isoform with the highest affinity for fibrin.

Key words

- Atherosclerosis
- Apolipoprotein(a)
- Thrombosis
- Atheroma
- Plasminogen
- Lipoprotein
- Fibrin
- Fibrinolysis

Introduction

Atherosclerosis is a complex silent process characterized by amorphous lipid accumulation in the intima, which may result in coronary heart disease. Atherosclerotic plaque rupture with superimposed thrombosis is present in coronary arteries in 80-90% of patients with fatal ischemic cardiac events

(1). Thrombosis results from an impaired antithrombotic response to vascular injury. Factors injurious to the arterial intima may alter its selective permeability, favor the accumulation of lipids with formation of fatty streaks, and allow deposition of micro-thrombi. Repair of early lesions is basically a healing process but beyond a certain stage the reparative process becomes largely pro-

liferative with multiplication of smooth muscle cells, macrophage recruitment with cholesterol deposition and organization of mural thrombi (2); repeated injury and an abnormal repair response contribute to the progression from early to advanced atherosclerotic lesions.

The lipoprotein(a) connection in atherosclerosis and thrombosis

Atherosclerosis is a multifactorial disease: high serum cholesterol concentrations carried by low density lipoproteins (LDL), high blood pressure and cigarette smoking have been established as major risk factors for coronary heart disease. A number of epidemiological and clinical studies have now established that high plasma concentrations of the lipoprotein Lp(a), an LDL-like particle discovered by Berg in 1963 (3), is also a major and independent risk factor for myocardial infarction. Lp(a) is a complex particle composed of a lipid core and two disulfide-linked subunits: apolipoprotein B-100 and apolipoprotein apo(a) (Figure 1). The lipid core and apo B-100 of Lp(a) are shared with LDL; in contrast, the apo(a) glycoprotein confers its characteristic properties on Lp(a). Apo(a) shows a high degree of homology with plasminogen, the precursor of the fibrinolytic enzyme plasmin.

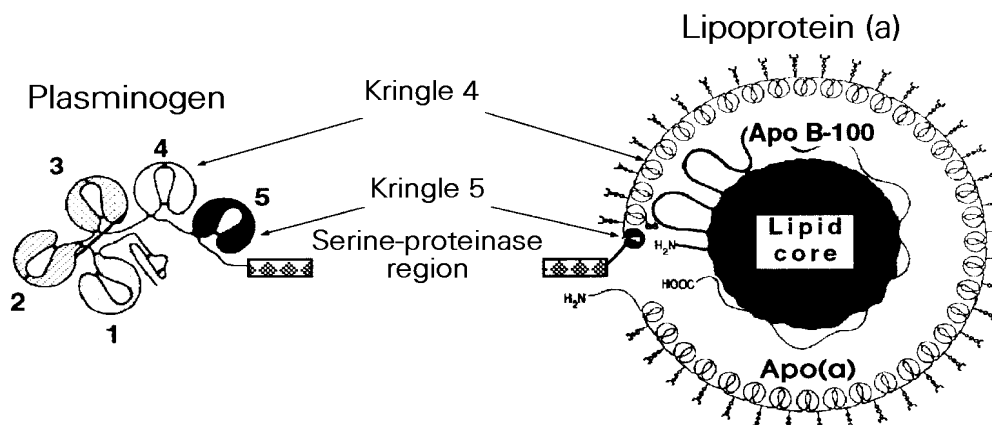
The mechanism by which Lp(a) may favor atherosclerosis is still a matter of debate

but the fact that Lp(a) has both LDL and plasminogen-like moieties suggests that Lp(a) may constitute a link between the processes of atherosclerosis and thrombosis. Indeed, Lp(a) and fibrin have been identified in atherosclerotic plaques (4-6); moreover, in transgenic mice expressing human apo(a), apo(a) co-localizes with lipid deposition on the arterial wall (7). In order to understand the basis of this connection we will briefly review the plasminogen activation system and consider recent evidence indicating that Lp(a) is a major risk factor for coronary heart disease.

Plasminogen and apolipoprotein(a): homologous proteins with opposite effects

Plasminogen is a single-chain glycoprotein of Mr 93,000 secreted by the liver and found in plasma at a concentration of 1.5 to 2 $\mu\text{mol/l}$. It consists of 791 amino acid residues arranged in two types of domains with functional autonomy: the kringle modules and the serine-proteinase region (Figure 1). Kringles are sequences of 80-90 amino acids arranged in a triple-loop tertiary structure rigidly stabilized by three disulfide bridges (8). The kringle structure was first described in prothrombin and is found in several copies in proteins that evolved from a common ancestral gene, i.e., plasminogen, apo(a) and hepatocyte growth factors. The kringle do-

Figure 1 - Structural homologies between apolipoprotein(a) and plasminogen. Lp(a) shares its lipid composition and apolipoprotein B-100 with low density lipoproteins (LDL). The characteristic feature of Lp(a) is the presence of a plasminogen-like apolipoprotein apo(a). This glycoprotein consists of a variable number of copies of kringle 4 and single copies of kringle 5 and the serine-proteinase region of plasminogen.



mains of plasminogen, designated 1 to 5, differ from each other and are connected to the proteinase domain by a sequence adjacent to the activation cleavage site Arg₅₆₁-Val₅₆₂. The serine-proteinase domain contains the active catalytic center (Ser₇₄₁, His₆₀₃, Asp₆₄₆) and is located in the carboxy-terminal region (Val₅₆₂-Asn₇₉₁), whereas the amino-terminal region (Glu₁-Arg₅₆₁) bears the five kringle domains and an amino-terminal peptide of 77 residues (Glu₁-Lys₇₇) that may be released by plasmin. Thus, native plasminogen possesses a glutamic acid as the amino-terminal residue (Glu-plasminogen), while the corresponding residue in the plasmin-cleaved form is lysine (Lys-plasminogen). Lys-plasminogen is not normally found in human plasma.

Kringles 1 and 4 of plasminogen contain a functional subsite supported primarily by amino acid residues of the inner loop. Since this subsite binds to lysine residues of fibrin and cell membrane proteins it has been termed lysine-binding site or LBS. The structure of this subsite, an ionic dipole with the anionic and cationic sites positioned at opposite ends of a hydrophobic trough, has been well defined (9). In both kringle 1 and kringle 4, the anionic center is constituted by Asp₅₅ and Asp₅₇ while the cationic center is mainly represented by Arg₃₄ and Arg₇₁ in kringle 1, and by Lys₃₅ and Arg₇₁ in kringle 4; the distance between Arg₇₁ and Asp₅₇ (6.8 Å) corresponds closely to the separation of the zwitterionic charges in lysine or in 6-aminohexanoic acid, a lysine analogue. The elongated hydrophobic depression of the kringle is lined with aromatic residues that

interact with the methylene groups of lysine located between the zwitterionic charges. The specific interactions between lysine residues in fibrin or cell membrane proteins and the lysine-binding subsites in kringles 1 and 4 of plasminogen allow plasminogen binding and activation.

Amino acid sequence analysis and cDNA cloning (10,11) have established that apo(a) contains a variable number of kringle domains that share 61-75% homology with kringle 4 of plasminogen. The kringle 4-like repeats of apo(a) are followed by a single copy of plasminogen kringle 5 and a protease domain that shares 94% homology with the corresponding domain of plasminogen. Kringle 4 copies of plasminogen in apo(a) are similar but not identical and have been classified into 10 different types (12) (Figure 2). Kringle 4 type 2 presents the lowest degree of homology with plasminogen kringle 4 and has no functional LBS; the number of this type of kringle in apo(a) is variable and gives rise to a series of apo(a) isoforms that contribute to the heterogeneity of Lp(a): a total of 34 apo(a) alleles and glycoproteins with molecular masses ranging from ~300 to ~800 kDa have been identified by protein (13) and cDNA (14) analysis. The other nine kringle types are present as single copies in all isoforms; kringle 4 type 9 possesses an additional cysteine residue that ensures the covalent binding between apo(a) and apo B-100 and thereby the formation of the Lp(a) particle. Sequence comparison and molecular modeling (15) have shown that a lysine-binding pocket similar to that of plasminogen kringle 4 is present in kringle

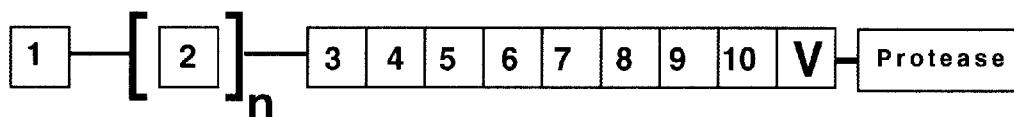


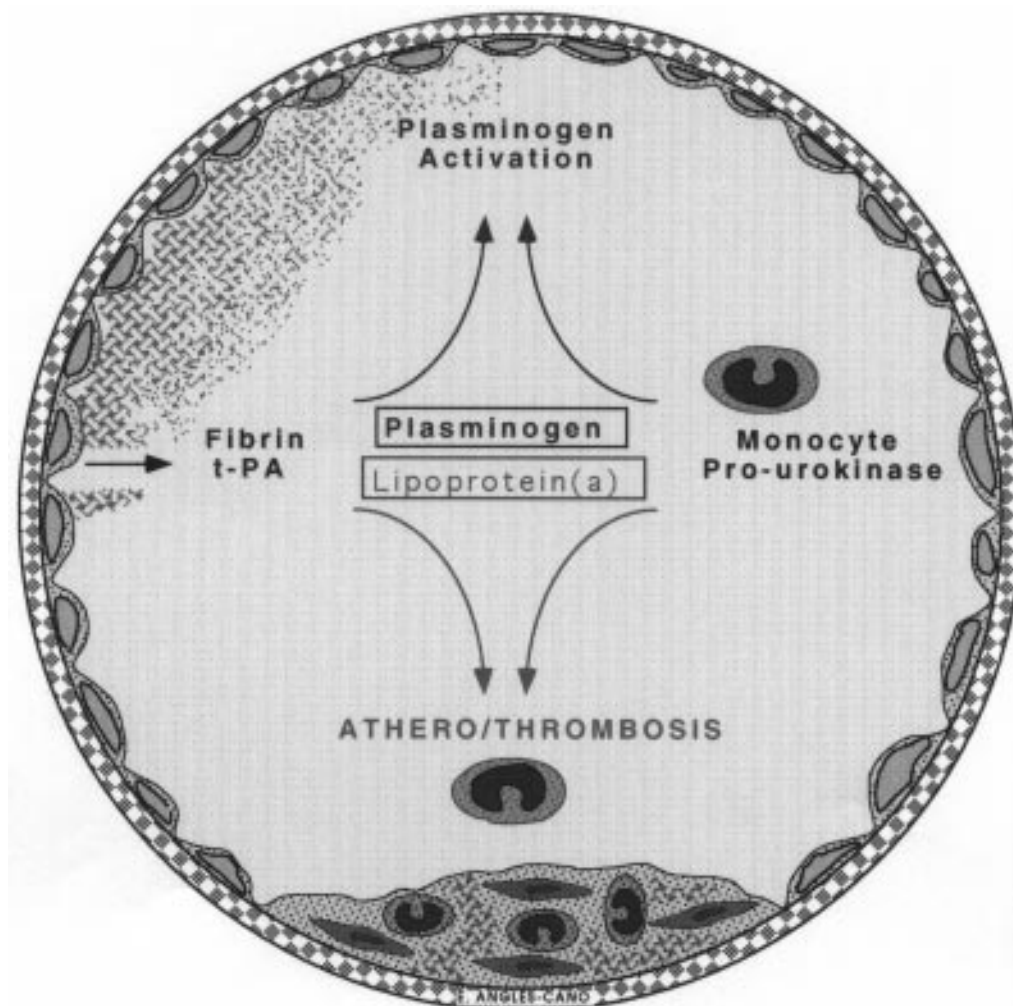
Figure 2 - Representation of the cDNA sequence of human apolipoprotein(a). The apo(a) gene contains a hypervariable region encoding a variable number ($n = 1$ to ~40) of kringles 4 type 2 that determines the existence of several apo(a) isoforms. The other kringle 4 types (1 and 3 to 10) and kringle 5 (V) are present as single copies. Some of these kringle 4 types endow apo(a) with lysine-fibrin binding properties similar to those of plasminogen.

4 type 10 of apo(a) (kringle 37 of the original apo(a) cloned by McLean et al. (10)) and that slightly modified LBS are present in kringle 4 types 5 to 8 (32 to 35 according to McLean et al.); these kringle copies may confer binding capabilities similar to those of plasminogen on apo(a). However, the Arg-Val residues of the activation cleavage site in plasminogen have been replaced by Ser-Ile in apo(a), a substitution that impairs recognition of apo(a) by plasminogen activators. Thus, binding of apo(a) instead of plasminogen to fibrin and cell surfaces may result in a diametrically opposed effect, i.e., inhibition of the generation of plasmin (Figure 3).

Inhibition of the generation of plasmin, the major mechanism of action of Lp(a)

Initial limited degradation of the surface of fibrin by plasmin unveils carboxy-terminal lysine residues and increases the local concentration of plasminogen, a process that amplifies and accelerates the degradation of fibrin. In a plasma milieu, the progression of such a process is markedly influenced by α_2 -antiplasmin, the specific plasmin inhibitor, which limits the number of carboxy-terminal lysine residues and thereby the amount of bound plasminogen (16,17). On the other hand, the blockade of such residues by iso-

Figure 3 - Plasminogen activation by t-PA on fibrin and by pro-urokinase on cell surfaces is the major mechanism of defense against thrombosis. Its inhibition by Lp(a) constitutes the major mechanism of the athero-thrombogenic effect of this lipoparticle.



lated plasminogen kringle 4 has been shown to interfere competitively with clot lysis by a mechanism involving binding to lysine-fibrin residues (18). Since the kringle domains behave as autonomous functional structures, the presence in apo(a) of kringle modules structurally related to those of plasminogen may result in analogous interactions with lysine residues of fibrin and cell membranes.

The effect of Lp(a) on plasminogen binding to fibrin and cell surfaces has been studied by several groups (19-23). A number of experimental *in vitro* studies resulted in convincing evidence that Lp(a) binds to the fibrin surface and cell membranes and thereby competes with plasminogen, inhibiting its activation (24,25). Such unique behavior was attributed to the fibrin-binding properties conferred by the kringle 4 repeats of apo(a) (26). Thus, Lp(a) interferes with the evolution of fibrinolysis on the surface of fibrin, endothelial cells, monocytes and platelets through binding of apo(a), an eternal zymogen that decreases the local concentration of plasminogen and cannot be transformed into an active enzyme. Most of the

effects of Lp(a) such as persistence of fibrin deposits, accumulation of cholesterol and proliferation of smooth muscle cells in the intima are related to a decrease in plasmin activity (Figure 3).

Hypofibrinolysis and cholesterol accumulation are a direct consequence of the presence of Lp(a) on the surface of fibrin and cell membranes: apo(a) inhibits plasmin formation and the LDL components favor cholesterol accumulation (Figure 4).

Growth and proliferation of vascular smooth muscle cells are inhibited by active TGF- β , a growth factor secreted in latent form and activated by plasmin (27). It has been recently shown that Lp(a) inhibits the generation of TGF- β (28) and that the generation of plasmin and thereby the activation of TGF- β are decreased in transgenic mice expressing human apo(a) (29). Insufficient activation of TGF- β may result in migration and proliferation of smooth muscle cells into the intima, an important mechanism in atheroma plaque formation. The pathophysiological importance of this mechanism *in vivo* in other animal models needs further confirmation.

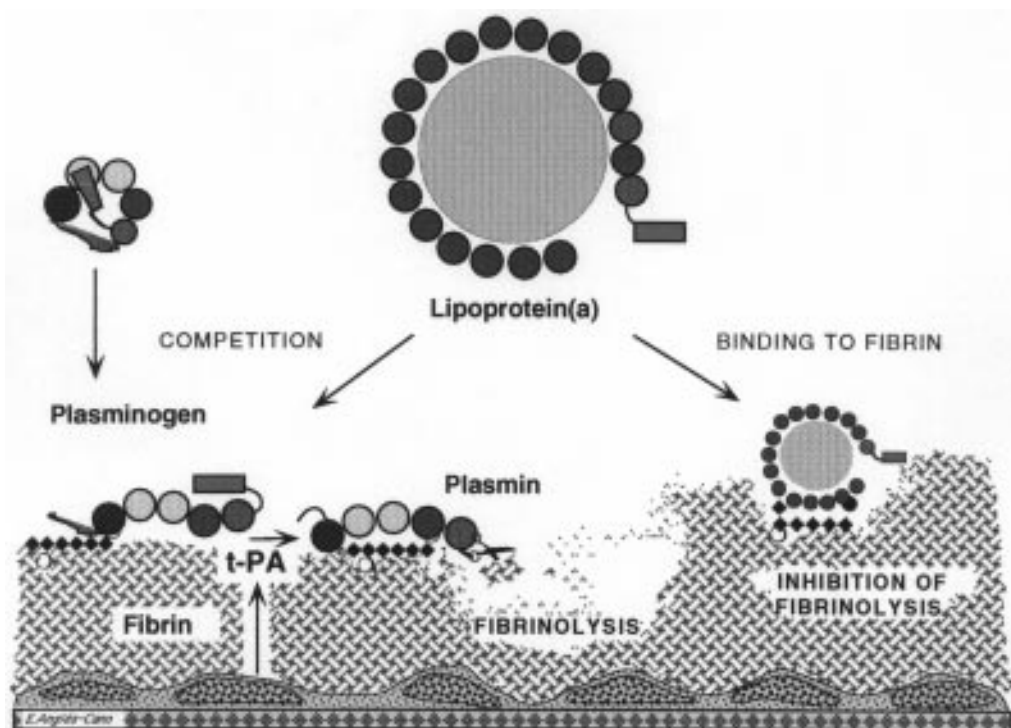


Figure 4 - Competitive inhibition of the binding of plasminogen by Lp(a). Inhibition of plasmin generation results in accumulation of fibrin, cholesterol and smooth muscle cells on the vascular wall.

Other athero-thrombogenic mechanisms of Lp(a)

Modification of protein synthesis. Lp(a) may stimulate the expression of PAI-1 and inhibit the synthesis of t-PA by endothelial cells in culture (30,31). Thus, inhibition of t-PA by PAI-1 and low t-PA antigen levels may enhance Lp(a)-dependent hypofibrinolysis by decreasing the amount of t-PA available for the activation of plasminogen.

Binding of Lp(a) to extracellular matrix components. Recent reports suggest that Lp(a) and recombinant apo(a) display high affinity for fibronectin and that Lp(a) may form complexes with proteoglycans or glycosaminoglycans of the extracellular matrix (32,33). These interactions are not related to the lysine-binding function of kringle 4 and may contribute to the accumulation of Lp(a) in the vascular wall.

Oxidation of Lp(a). The Lp(a) and LDL particles are sensitive to oxidative processes. Phagocytosis of oxidized Lp(a) and LDL particles results in the formation of foam cells (34,35). Antioxidants such as probucol

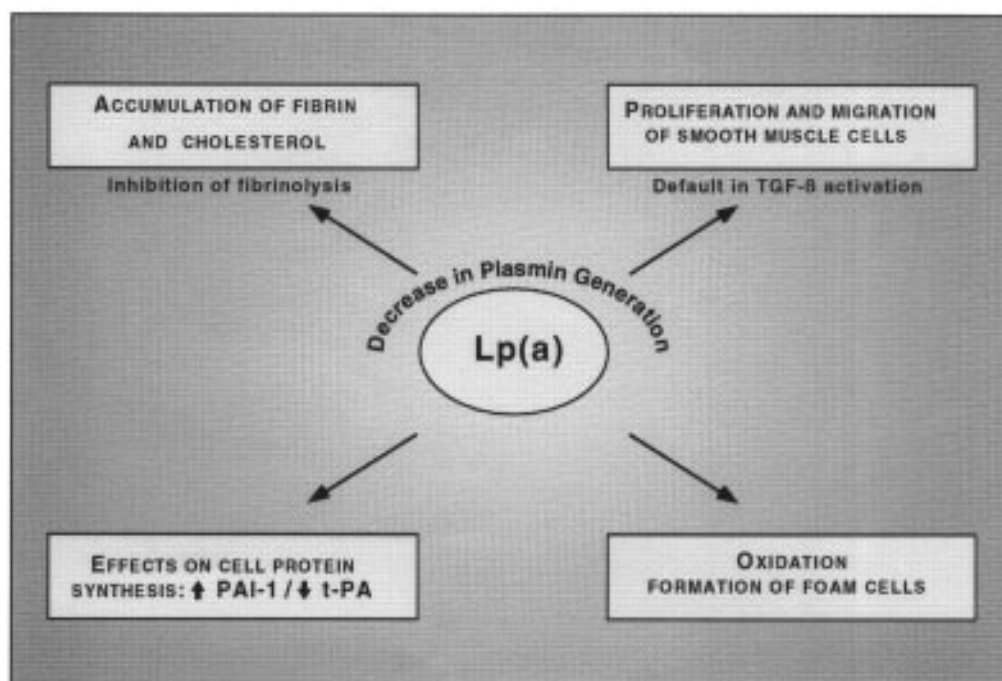
and vitamins C, E and β -carotenes may prevent such reactions.

Figure 5 represents an overall view of these different mechanisms.

Antifibrinolytic activity of apolipoprotein(a) *in vivo*

Most clinical studies have failed to demonstrate that high levels of Lp(a) decrease systemic fibrinolysis (36,37). To date there is no definitive evidence that Lp(a) interferes with plasminogen binding and activation *in vivo*. Scarce evidence has been obtained from a few experimental studies. Occlusive arterial thrombosis with incorporation of Lp(a) into damaged arterial segments was observed in cynomolgous monkeys with high plasma Lp(a) levels (38). Biemond et al. (39) studied the effect of a recombinant form of apo(a) on endogenous and t-PA-mediated lysis in an *in vivo* model of experimental thrombosis; endogenous thrombolysis but not t-PA-induced lysis was significantly reduced in the presence of apo(a). In contrast, transgenic mice that express hu-

Figure 5 - General scheme representing different modes of action of Lp(a) in the vascular wall. Inhibition of plasmin generation may be further enhanced by modifications of the synthesis of t-PA and PAI-1. Oxidized Lp(a) may contribute to the formation of foam cells by macrophages.



man apo(a) have been shown to exhibit reduced t-PA-induced lysis of pulmonary emboli produced by injection of human platelet-rich plasma clots (40). However, in these experiments the role of PAI-1 in the inhibition of the reduced doses of t-PA used was not ruled out. These studies strongly suggest that apo(a) may decrease fibrinolysis *in vivo*. The existence of such a potential pathophysiological mechanism and its relevance to the development of athero-thrombosis are supported by the fact that high Lp(a) levels associated with cardiovascular disease are genetically determined.

Genetic polymorphism and functional heterogeneity of Lp(a)

The absence of clinical or biological signs of disease in the presence of low or undetectable levels of Lp(a), and the existence of an important polymorphism of apo(a) indicative of a low pressure selection suggest that Lp(a) may not be a vital biological factor. In contrast, a number of retrospective studies have shown that high circulating levels of this lipoparticle are associated with a high risk for cardiovascular and cerebrovascular diseases in young adults (<60 years old). Recent prospective studies have confirmed these observations (41-45) (Table 1). It is now well recognized that the circulating concentration of Lp(a) is mainly regulated by the apo(a) gene (46). The size of each allele varies as a function of the number of repetitive sequences encoding kringle 4 type 2 (a total of 34 isoforms have been identified by cDNA (14) and protein (13) analysis). In general, the smaller this hypervariable region and therefore the size of the apo(a) isoform, the higher the plasma concentration of Lp(a). The question is therefore to know whether the cardiovascular risk associated with Lp(a) is linked to apo(a) isoforms of low molecular mass. Recent experimental and clinical evidence provides arguments favoring this hypothesis.

Table 1 - Lipoprotein(a) in coronary heart disease: prospective studies.

N = Number of subjects; case/controls.

No correlation	
Helsinki Heart Study (58)	(N = 4081; 138/130)
Physician's Health Study (57)	(N = 14916; 296/296)
Positive correlation	
Göteborg Study (41)	(N = 776; 26/109)
British United Provident Association Study (43)	(N = 21520; 229/1145)
Lipid Research Clinics (44)	(N = 3806; 233/390)
Göttingen Study (42)	(N = 5471; 214/5124)
Framingham Heart Study (45)	(N = 1340; 169/1171)

Indeed, a difference in the distribution of apo(a) isoforms between patients with atherosclerosis and a control population has been recently reported (47-49). Low molecular mass isoforms (B, S1 and S2 according to the nomenclature of Utermann (49)) were found more frequently in subjects with high Lp(a) concentrations and a history of myocardial infarction or intermittent claudication. Thus, short apo(a) alleles may favor atherogenesis by increasing the concentration of Lp(a). However, a difference in allele distribution between patients at risk and controls is not always observed, and the inverse relationship between apo(a) size and Lp(a) concentration is not linear, thus suggesting the existence of a functional diversity among apo(a) isoforms. Indeed, some plasmas with a high Lp(a) concentration may fail to induce a decrease in fibrinolysis (50). In such case, at similar concentrations of plasminogen and Lp(a), it is the functional behavior of apo(a) that may determine the inhibition of plasminogen binding.

Since the atherogenic potential of Lp(a) is related to the lysine-binding properties conferred by the kringle 4 repeats of apo(a), we have recently explored the possibility that Lp(a) phenotypes may have different functional properties with regard to their affinity for fibrin. We demonstrated that Lp(a) particles from homozygous subjects containing a single, distinct isoform of apo(a) dis-

play different affinities for fibrin as a function of their size; this finding suggests that the variable number of kringles in apo(a) influences its ability to bind to fibrin (51). Thus, isoforms of low molecular mass showing the highest affinity for fibrin are the best competitor for plasminogen and are epidemiologically related to a higher cardiovascular risk (49-51). The main structural difference between isoforms almost certainly reflects a variable number of kringle 4-like repeats of type 2; kringle 4 type 10 appears to contain a lysine-binding subsite similar to that present in plasminogen kringle 4, and kringles type 5 to 8 possess a slightly modified LBS. We have therefore suggested that due to kringle-kringle interactions, an increasing number of kringles may render the specific lysine-binding subsite in apo(a) inaccessible to fibrin. Scanu et al. (52) have recently proposed that point mutations in kringle 4 type 10 may occur in humans and that phenotypes with the same number of kringle 4 repeats may be functionally different in terms of their thrombogenic potential; however, their frequency in the general population is very low (<2%). Whether the marked variation in apo(a) binding properties is due to the presence or absence of specific kringle sequences or to a variable number of kringles needs further investigation. Taken together, these results suggest that, in addition to the quantitative factor, an important qualitative effect must be considered in the athero-thrombogenic role of Lp(a).

Binding of Lp(a) to fibrin and the prediction of Lp(a) as a risk factor for cardiovascular disease

The plasma concentration of Lp(a) is determined by genetic factors and remains relatively constant throughout life; dietary changes or lipid lowering drugs have no effect on Lp(a). However, Lp(a) levels may increase after alcohol withdrawal, in the

nephrotic syndrome and during acute phase reactions; also, different hormone-dependent mechanisms may modulate plasma Lp(a) concentration in man. Lp(a) levels vary widely among individuals (from <1 mg/dl to more than 1 g/l) and among different populations (higher levels of Lp(a) have been demonstrated in blacks). The definition of a threshold risk value for Lp(a) is therefore complicated by such variations and by the methodological problems encountered with the techniques for Lp(a) determination and with the lability of the Lp(a) lipoprotein particle (53). However, a so called "normal" level of Lp(a) has been arbitrarily assigned using a cut-off point of 20 to 30 mg/dl, most probably based on the highly skewed distribution of Lp(a) concentration towards low levels in the general population. Based on this value, it has been established that high Lp(a) concentration is a major independent risk factor for acute myocardial infarction in individuals less than 60 years old (41,54,55). However, a few retrospective (56) and prospective (57,58) studies have failed to detect a correlation between high Lp(a) levels and coronary heart disease. The inadequacy of some test techniques to correctly quantitate Lp(a), the fact that most subjects are heterozygous for the apo(a) trait and that Lp(a) particles having a given apo(a) isoform may display varying concentrations, may offer an explanation for these conflicting results. Our findings of an inverse relationship between isoform size and affinity for fibrin, however, raise the hypothesis that *the real risk factor is the Lp(a) population with high affinity for fibrin* (59). According to this concept, some Lp(a) phenotypes may not be related to atherogenesis and, therefore, some individuals with high Lp(a) would not be at risk of coronary heart disease. This new concept of the functional heterogeneity of Lp(a) adds a new dimension to the evaluation of the predictive value of Lp(a) as a risk factor for cardiovascular disease.

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