review

Learning about the structure and biology of human lipoprotein [a] through dissection by enzymes of the elastase family: facts and speculations

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Abstract Lipoprotein[a], Lp[a], represents a class of lipoprotein particles that have as a protein moiety apoB-100 linked by a disulfide bridge to a multi-kringle structure, apolipoprotein[a], or apo[a]. It is now possible to separate from Lp[a] a free apo[a] able to reassociate with apoB-100-containing lipoproteins to restore the parent lipoprotein complex. Apo[a], whether free or a constitutive component of Lp[a], can be cleaved at interkringle sites by the action of enzymes of the elastase family generating fragments that differ in structural, functional, and metabolic properties. In the case of Lp[a], elastase digestion generates a miniLp[a] particle, which contains the apo[a] COOH-terminal domain able to bind to lysine, fibrinogen, fibronectin, and proteoglycans. This domain may also be generated by elastase type enzymes secreted by activated macrophages and smooth muscle cells in the arterial intima as a part of the chronic inflammation that characterizes the atherosclerotic process. Thus, the apo[a] immunoreactive material, which has been described in the atherosclerotic plaque, may represent miniLp[a] and/or apo[a] fragments accumulating in the vessel wall as a function of their relative affinity for the components of the extracellular matrix and producing complexes with an atherothrombogenic potential. This potential may depend on several factors: kringle folding and conformation, susceptibility of the linkers to proteolytic cleavage, binding specificity of given apo[a] fragments to the matrix components of the arterial intima, and the overall inflammatory status of the arterial wall.—**Scanu, A. M., and C. Edelstein.** Learning about the structure and biology of human lipoprotein[a] through dissection by enzymes of the elastase family: facts and speculations. *J. Lipid Res.* 1997. **38:** 2193–2206.

Supplementary key words miniLp[a], • apolipoprotein[a] • fragments • PMN elastase • inflammation and atherosclerosis

INTRODUCTION

The discovery of a marked degree of homology between apo[a] and plasminogen (1) ignited the field of Lp[a] research and stimulated numerous studies which led to important advances in the areas of genetics, structure, and function of this unique, highly polymorphic lipoprotein variant (2, 3). Significant work has also been conducted on elucidating the mechanisms underlying the relationship between the cardiovascular pathogenicity and high plasma levels of Lp[a] (4) although many questions still remain unanswered (2, 5).

Lp[a] comprises a heterogenous class of lipoprotein particles containing apo[a], the characteristic glycoprotein of Lp[a] (1, 2). Although much progress has been made, acquisition of knowledge has been hampered by the fact that apo[a] in the plasma is mostly found as a constitutive component of Lp[a] (6). Early attempts to dissociate apo[a] from Lp[a] using reducing agents have resulted in a non-functional product, likely because the targeted disruption of the interchain disulfide between apo[a] and apoB-100 resulted in intrachain disulfide cleavages compromising the stability of the kringle domains (3, 5). Consequently, the information on the function of free apo[a] has been derived mainly from the study of recombinants (7) which, although very useful, have not been unequivocally proven to represent true native apo[a] in terms of fidelity of folding, conformation, and carbohydrate composition. Recent studies from this laboratory have demonstrated that a free, functional apo[a] can be dissociated from Lp[a] under mild reductive conditions (8). Such a product has proven to be critical in demonstrating that its functional properties differ significantly from those of apo[a] when examined as a constitutive member of Lp[a] (9). In addition, several investigators have reported the presence of structures smaller in size than apo[a], referred

Abbreviations: Lp[a], lipoprotein[a]; apo[a], apolipoprotein[a]; LDL, low density lipoprotein; PMN, polymorphonuclear cell(s); K, kringle; EACA, ε-aminocaproic acid; LPBD, lys-pro binding domain.

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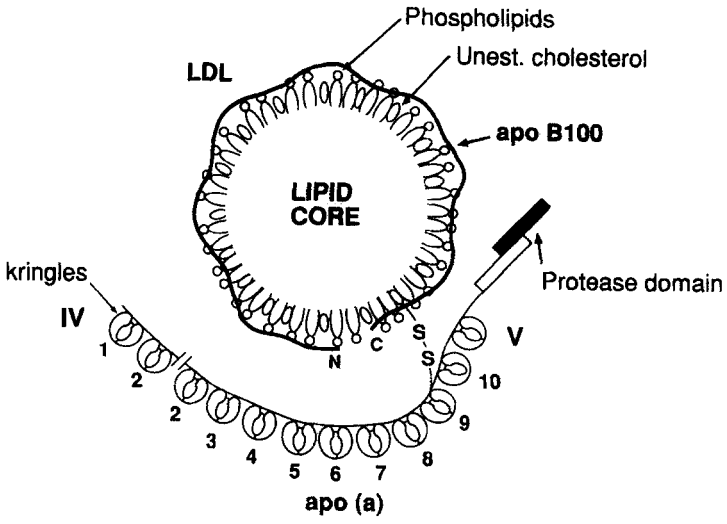


Fig. 1. Schematic drawing of Lp[a] showing an LDL particle wrapped around by apoB-100 linked in the COOH-terminal domain by a single disulfide bridge to apo[a]. The latter is depicted as multiple kringles comprising two types classified as IV and V in view of their close homology to kringles 4 and 5 of plasminogen. There are several kringle IV-2 repeats; all of the other kringles occur as a single copy. Kringle IV-9 is unique because it contains an unpaired cysteine residue in position 4057 engaged in disulfide linkage with Cys4326 located in the COOH-terminal domain of apoB-100. (Modified from ref. 89.)

to as "fragments," in the plasma (10) urine (11, 12), and atherosclerotic plaques of human subjects (13). However, those studies provided no insight into the origin and biological relevance of these fragments.

The purpose of this article is to discuss the apo[a] fragments in the context of the recent developments in the field with a view on Lp[a] as a multi-kringle/linker domain structure amenable to dissection into smaller functional units by elastases, biologically relevant enzymes of the serine protease family.

LP[a]

Lp[a] represents a class of lipoprotein particles having a core of neutral lipids and a protein moiety containing one mole of apoB-100 covalently linked by a disulfide bond to one mole of apo[a], the specific glycoprotein component of Lp[a] (**Fig. 1**). The lipid core can be either triglyceride (TG)- or cholesteryl ester (CE)-rich and referred to as TG-Lp[a] and CE-Lp[a], respectively. Most of the Lp[a] particles in the circulation are of the latter type and are the ones most extensively investigated in structural and functional studies. Lp[a] is heterogenous in size and density due to variations in lipid content and apo[a] size (14–16). Moreover, about 94% of human subjects have two apo[a] size isoforms (17) and as there is only one size isoform per particle, those subjects should have only two classes of Lp[a] particles. However, from the technical standpoint they would be difficult to seperate unless a significant differential exists in the size of the two apo[a]s. In general, particle heterogeneity makes it difficult to isolate Lp[a] from the other plama lipoproteins by density criteria only. Thus, size exclusion (18) or ion exchange chromatography is usually required as complementary methods to ultracentrifugation along with a lysine-Sepharose affinity chromatography step in subjects with a lysine binding competent Lp[a].

FREE APO[a]

General structure

The amino acid sequence of apo[a] has been inferred from its cDNA sequence (1) and found to exhibit a high degree of homology to human plasminogen. **Diagram A** illustrates the essential features of the amino acid sequence indicating the positions of the kringles and linkers. Nascent apo[a] contains a 19-residue signal peptide that is released during processing to yield a mature protein which has glutamine at the amino terminus and eleven triple-loop structures each stabilized by three disulfide bonds termed kringles (K) (19). Apo[a] contains up to 54 kringles which due to their homology to those present in plasminogen have been classified as types IV and V (3). All of them are present as a single copy except for kringle IV-2 which by occurring in identical repeats determines the size of apo[a] and influence the levels of Lp[a] in the plasma (16, 20–23). The numbering system in Diagram A is based on the original cDNA sequence containing 28 KIV-2 repeats. The eleven types of kringles are each coded by two separate exons with introns inserted at positions as in the plasminogen gene, i.e., in the middle and at both ends of each kringle (24). All the kringles except KIV-1, KIV-2, and KV are 77 amino acid residues in length. KIV-1 and KIV-2 contain 78 amino acids and KV has 79. The interkringle regions (linkers) each have 36 amino acids except for the linkers connecting KIV-6 to KIV-7 and KIV-10 to KV containing 28 and 26 amino acids, respec-

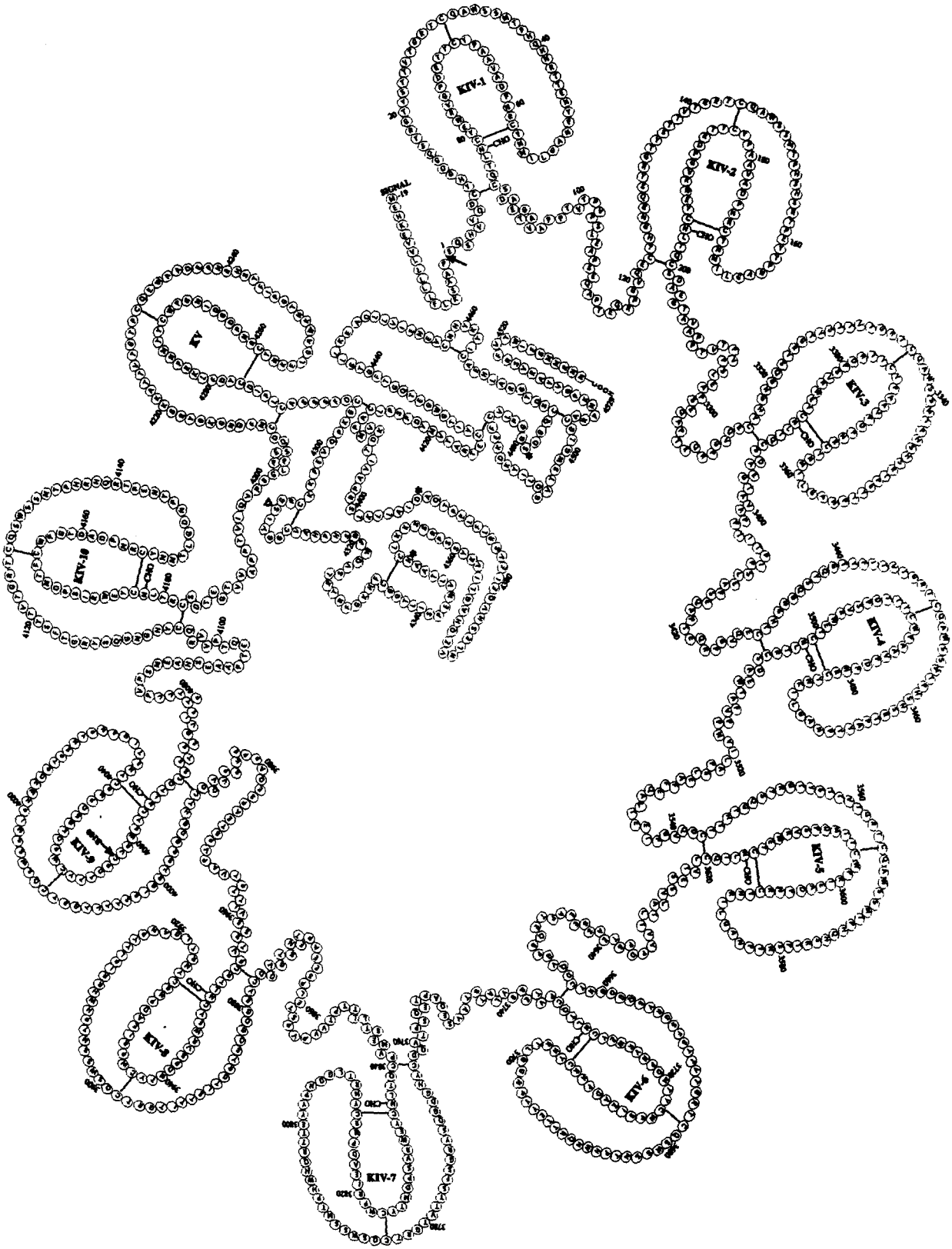
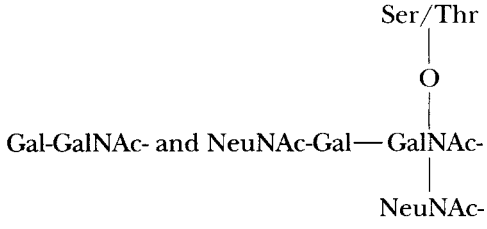


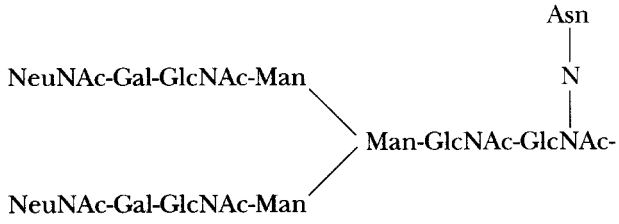
Diagram A. Schematic display of kringles and linkers from the amino acid sequence data deduced from the analysis of the apo[a] cDNA (1). Only one copy of the kringle IV-2 repeat is shown. The arrangement of the linkers is arbitrarily drawn and has no structural basis. To the left of KIV-1 is the 19-residue signal peptide. The arrow indicates the first amino acid of the mature apo[a]. A second arrow in kringle IV-9 shows the position of the unpaired cysteine involved in disulfide linkage with apoB-100. The position of the N-linked carbohydrates are denoted as CHO on each kringle.

tively. The COOH-terminal domain of apo[a], which begins with residue 4295, exhibits a high degree of homology with the serine protease region of human plasminogen. This region contains the catalytic triad, His4350–Asp4393–Ser4481, (marked by asterisks) which is typical of serine protease enzymes. This triad is also present in plasminogen where it becomes active upon cleavage of the Arg561–Val562 bond. This activation does not occur in apo[a] because the Arg required for activation is replaced by Ser4308 (1) (marked by an inverted open triangle). The fact that the splice junctions of the 6 exons coding for the protease domain of apo[a] are located in the same positions as in the plasminogen gene (24, 25) and that each of the three amino acid residues in the catalytic triad are coded by a separate exon (24) lend support to the hypothesis that apo[a] and plasminogen are derived from a common ancestor gene through a series of duplications and exon shufflings (1, 26, 27).

In each kringle, the cysteines which are involved in the formation of the three intrachain disulfide bonds, are conserved. KIV-9 contains, in addition, an unpaired cysteine in position 4057, the site where apo[a] forms a disulfide bridge with Cys 4326 of apoB-100 in Lp[a] (28). In the apo[a] isoform under study, Fless, ZumMallen, and Scanu (29), found approximately 28% carbohydrates by weight in a molar ratio of 3:7:5:4:7, mannose:galactose:galactosamine:glucosamine:sialic acid, respectively. Moreover, based on the knowledge of the molecular weight of their apo[a] preparation, its high content in sialic acid, galactose, galactosamine and absence of fucose, they predicted a content of 36 O-linked oligosacharides with the structure:



and from the carbohydrate molar ratios, the existence of 14 N-linked oligosaccharides arranged as follows:



The existence of different structural domains suggests that apo[a] may exhibit differences in carbohydrate content and composition within and among size isoforms. This heterogeneity may be related to the number of glycosylation sites along the polypeptide chain, oligosaccharide chain length, oligosaccharide branching patterns, or a combination of these possibilities. Each KIV contains one possible N-linked glycosylation site at the sequence Asn–Leu–Thr (marked by CHO in Diagram A. This site is not present in KV or in the protease region. The O-linked glycosylation sites are mainly located in the linkers and therefore distributed over a large portion of apo[a] imparting a high degree of hydrophilicity.

Kringles of apo[a]

Kringles not only occur in apo[a] and plasminogen but also in other proteins of the fibrinolytic and coagulation systems (3, 19, 30). In apo[a], the kringles contain 77 to 79 amino acids and are thought to play an important role in regulating the interaction of the parent protein with its ligands. Kringles are involved in interactions with small molecules. For example, in apo[a] the KIV-10 and the region encompassing KIV-5 to KIV-8 have a high binding affinity for lysine, lysine analogs, and fibrin(ogen) (8, 9, 31–33). All of the reported types of kringles of apo(a) are presented in Fig. 1 and in Diagram A.

Linkers

The kringles of apo[a] are joined by linkers whose function is still largely undetermined. It is likely, however, that they influence the structural flexibility of the kringle domains and the overall activity of apo[a]. The specificity of function of these linkers is suggested by the amino acid sequence data inferred from the analysis of apo[a] cDNA (1). Contrary to the linkers joining the identical kringle IV-2 repeats, those between non-identical kringles differ in the length of the peptide chain (26 to 36 amino acid residues) and composition. Moreover, the linkers are predicted to be heavily glycosylated. According to predictions from the amino acid sequence data (34), O-glycosylation is the predominant mode of carbohydrate linkage involving both the threonine and serine residues. The linkers of apo[a] and their predicted O-glycosylation sites (marked by an asterisk) are presented in **Fig. 2.** Linker 4, which also contains the primary cut site by elastase (see below), is not predicted to have glycosylation sites. In contrast, linker 7 is highly O-glycosylated and also contains one N-glycosylation site.

LINKER				
1	SDAEGTAVAP	PTVTPVPSLE	APSEQAPTEQ	RPGVQE
2	SDAEGTAVAP	PTVTPVPSLE	APSEQAPTEQ	RPGVQE
3	SDAEGTAVAP	PT I TP IPSLE	APSEQAPTEQ	RPGVQE
4	SDAEWTAFVP	PNVILAPSLE	AFFEQALTEE	T PGVQD
5	LVT E SSVLAT	LTVVPDPSTE	E SSEEAPTEQ	SPGVQD
6	PVT E SSVLAT	STAV	. . SEQAPTEQ	SPTVQD
7	PVME STLLTT	PTVVPVPSTE	L P SEE APTEN	STGVQD
8	PV TE SSVLTT	PTVAPVPSTE	APSE QAP PEK	SPVVQD
9	SE TE SGVLET	PTVVPVPSME	AHSEAAPT EQ	TPVVRQ
10	SDTE GTVVAP	PTV IQVPSL G	PPSEQD	. . . . .

Fig. 2. Representation of the 10 linkers of apo[a] with the predicted O-glycosylation sites marked with an asterisk. The linker is identified according to the lowest number of the two kringles it connects.

Functional free apo[a]

Recent studies have shown that a functional free apo[a] can be obtained by subjecting Lp[a] to a mild reductive procedure using DTE to permit the cleavage of the interchain disulfide bond between apo[a] and apoB-100 without affecting the structural integrity of the individual kringles (8). The reaction is conducted in the presence of ε-amino caproic acid, EACA, a lysine analogue, in order to prevent the re-association of the cleaved products, apo[a] and apo[a]-free Lp[a]. The apo[a] obtained under these experimental conditions is able to bind to lysine–Sepharose, fibrinogen, and fibronectin and re-establish a disulfide linkage with the apoB-100-containing lipoproteins (8). Thus, by selecting appropriate in vitro conditions, it is possible to readily effect the disassembly and re-assembly of Lp[a]. Those studies have also shown that the reassociation of apo[a] with apoB-100-containing lipoproteins is prevented by the presence of either EACA or proline or both. Of interest, Lp[a] reassembly is independent of the functional state of the high affinity lysine binding site (LBS) located in apo[a] kringle IV-10, a site that is responsible for the binding of Lp[a] to lysine–Sepharose (9, 35). In turn, the in vitro reassembly of Lp[a] requires the activity of the low affinity domain comprising kringles IV-5 to IV-8 which we have referred to as the lys-pro binding domain (LPBD). In the Lp[a] particle this site is either partially or totally masked and can be unmasked by either the action of a detergent on Lp[a] or by dissociating apo[a] from the Lp[a] complex. This explains why Lp[a] species that are lys^- because of a nonfunctional high affinity LBS, can generate a lys^+ free apo[a] upon exposure of a functional LPBD (8, 9).

DISSECTION OF APO[a] AND LP[a] INTO STRUCTURAL AND FUNCTIONAL DOMAINS BY PURIFIED ENZYMES OF THE ELASTASE FAMILY

The fact that Lp[a] is large and heterogeneous has made it difficult to study its properties by conventional means. Thus, an important development in the field has been the finding that enzymes of the elastase family, and in particular porcine pancratic and human leukocyte elastase, can effect the cleavage of this lipoprotein into smaller units amenable to structural and functional analyses (36, 37). As a serine protease, the reactive site of elastase contains the catalytic triad His–Asp–Ser where a charge–relay system causes His and Asp to transiently bind a proton from the serine residue. This, in turn, becomes a highly reactive nucleophile capable of attacking susceptible peptide bonds of the target proteins and particularly smaller uncharged relatively bulky side chains like Val and Thr (38). Leukocyte elastase is abundant in peripheral blood polymorphonuclear cells (PMN) and is stored in cytoplasmic azurophilic granules from which it is rapidly released upon cell activation. The synthesis of the enzyme does not occur in adult PMN rather in bone marrow precursors about the time of their commitment to the myelocytic series (39, 40).

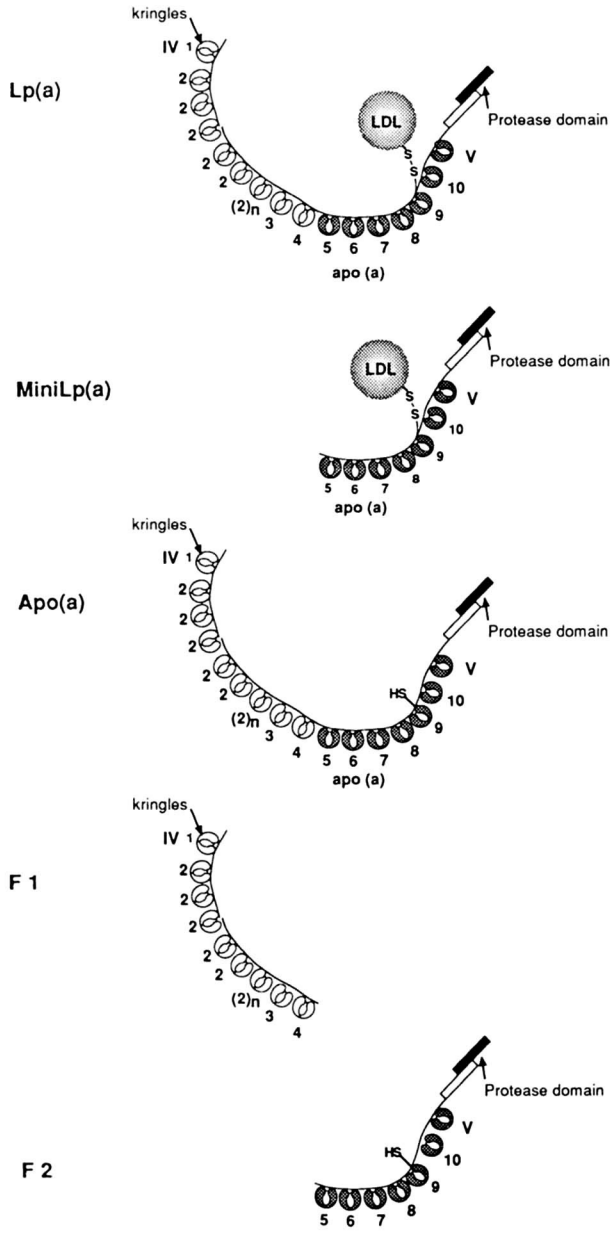


Fig. 3. Schematic diagram of the structure of Lp[a], miniLp[a], apo[a], F1, and F2. The figure is not drawn to scale and is meant to emphasize the main products obtained from the digestion of Lp[a] and apo[a] by elastase. (Reprinted from ref. 36, with permission.)

Studies on free apo[a]

Free apo[a] when subjected to limited proteolysis by a pure preparation of porcine pancreatic elastase, undergoes cleavage at the lle3520–Leu3521 bond situated in the linker region between kringles IV-4 and IV-5. Such a cleavage generates two main fragments, one representing the NH_2-terminal domain, called F1 and the other the COOH terminal domain, called F2 both exhibiting distinct structural, functional and metabolic behaviour (36) (**Fig. 3**). F1 consists of kringles IV-1, IV-2 repeats, IV-3, and IV-4 and appears to be functionally inert. Moreover, the size of F1 varies according to the apo[a] size isoform which is dependent on the number of KIV-2 repeats. F2 is composed of KIV-5 through the protease region and binds to lysine–Sepharose, fibrinogen, and fibronectin and forms a miniLp[a] particle when incubated with LDL. The size of F2 is constant and, based on amino acid composition, is calculated to be 113 kDa. The apparent size estimated by gel electrophoretic criteria is 170 kDa due to the fact that this domain is highly glycosylated.

Metabolic studies in normal mice have shown that intravenously injected F1 is rapidly cleared ($T_{1/2}$, 2.9 h) and appears in the urine after 1 h as smaller fragments in the size range of 100 to 33 kDa (36). In contrast, F2 has a longer residence time ($T_{1/2}$, 5 h) and is excreted in markedly lower amounts in the urine after 5 h as fragments of 70–45 kDa. Based on ELISA quantitation, the F1 and F2 fragments in the urine represent less than 0.5% and 0.05%, respectively, of the injected material. It is interesting to note that the $T_{1/2}$ of apo[a] is identical to that of the unfractionated apo[a] digest (3.7 h), suggesting that the mouse is able to cleave apo[a] in a manner comparable to that elicited by elastase in vitro. It should also be stressed that the metabolic studies on human Lp[a] and derivatives injected into the mouse may not be extrapolated to man since the mouse lacks apo[a] and may not catabolize this protein and its fragments in the same way as human subjects do.

When apo[a] is subjected to proteolysis by incubation with purified leukocyte elastase (37), the major cut is still at the interkringle region between KIV-4 and KIV-5 (lle3520–Leu3521). However, other cleavages occur between KIV-7 and IV-8 (Thr 3846–Leu 3847) and between kringles IV-10 and V (lle 4196–Gin 4197). Thus, limited digestion of apo[a] by leukocyte elastase can cause the formation of at least seven fragments, F1 (KIV-1 to KIV-4); F2 (KIV-5 to protease region); F3 (KIV-5 to KIV-10); F4 (KIV-8 to protease region); F5 (KIV-8 to KIV-10); F6 KIV-5 to KIV-7) and F7 (KV to protease region) of which only F1 and F7 lack lysine-binding capability (**Fig. 4**). Of interest, the cleavage pattern by elastase is unaffected by apo[a] size polymorphism, i.e., independent of the number of KIV-2 repeats.

Studies on Lp[a]

As in the case of apo[a], elastase cleaves Lp[a] and generates F1 and F2 (36). However, F2 is linked to LDL in the form of an LDL-F2 complex which we have called miniLp[a] in accordance with the classification of Huby et al. (41, 42) who reported the generation of a mini-Lp[a] particle by subjecting Lp[a] to limited digestion with thermolysin. This enzyme, like elastase, cleaves

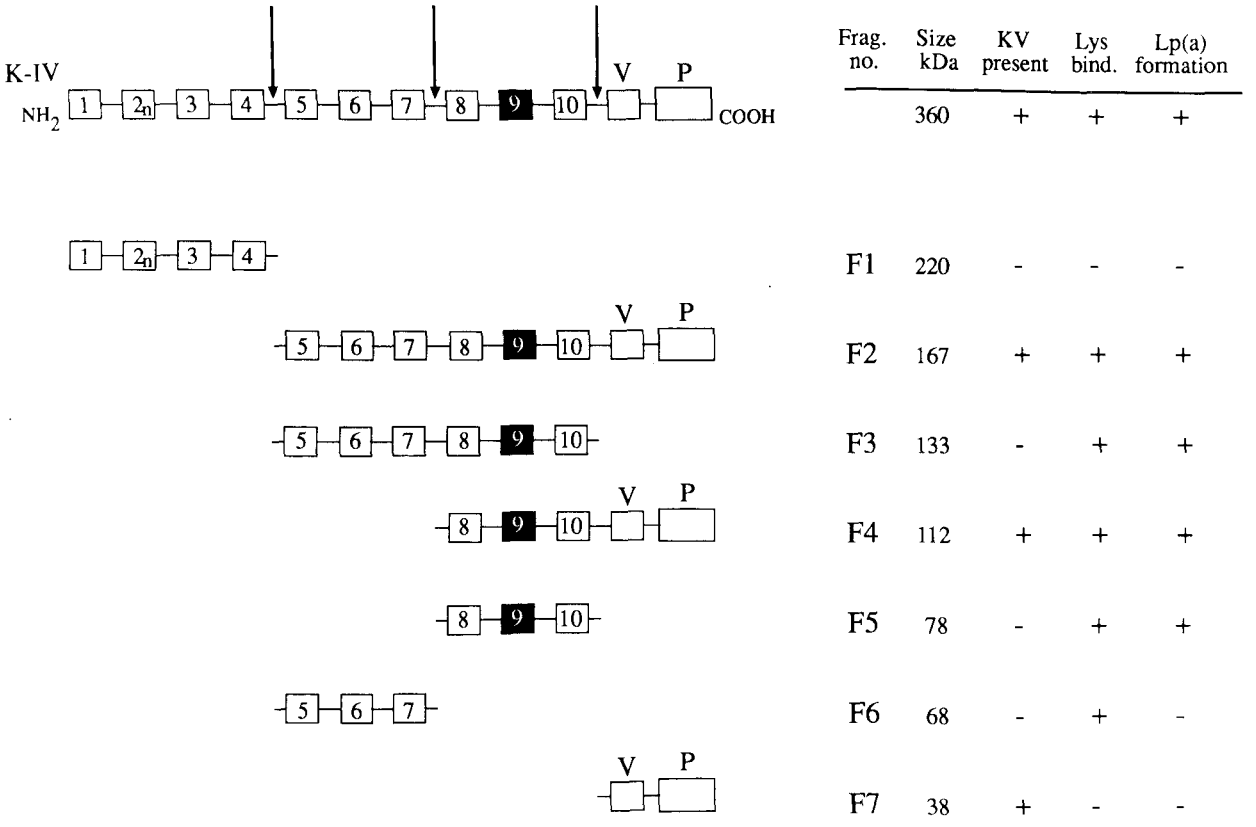


Fig. 4. Properties of the fragments of apo[a] obtained by elastase digestion. The fragments are presented on the left panel. The right panel summarizes their properties in terms of binding to lysine—Sepharose, apparent size from electrophoretic data, and the presence of KV by immunoblot analyses. The black squares indicate the position of KIV-9. (Reprinted from ref. 37, with permission).

apo[a] in the linker region between kringles IV-4 and KIV-5 but at a different site, namely at the Ala 3513–Phe 3514 bond, which is seven amino acids upstream of the cleavage site by elastase. The elastase-generated miniLp[a] containing the F2 fragment, binds to lysine–Sepharose, fibrinogen and fibronectin as would be predicted by the presence in this fragment of both the high affinity (KIV-10) and the low affinity (KIV-5 to KIV-8) binding sites. Turnover studies in mice have shown that this miniLp[a] has an apparent $T_{1/2}$ of 8.3 h which is significantly longer than that of whole Lp[a] ($T_{1/2}$, 5.1 h), F1, F2 and apo[a] indicating an effect of apo[a] truncation on the catabolism of Lp[a] by mechanisms yet to be determined (36).

Multi-site fragmentation of Lp[a] also occurs upon limited digestion with purified leukocyte elastase (37). The fragmentation occurs at three sites and is the same whether free apo[a] or Lp[a] is studied indicating that the covalent attachment of apo[a] to apoB-100 in Lp[a] does not hinder the elastase action. Lp[a] fragments containing kringle IV-9 (Figs. 3 and 4) remain linked to apoB-100 via the disulfide bond and form miniLp[a] particles in which the size of apo[a] varies according to the size of the fragments produced by the elastase digestion. Purified PMN elastase also causes a partial digestion of apoB-100 without an apparent destabilization of these particles (37).

MODIFICATION OF LP[a] AND APO[a] UPON INCUBATION WITH PMN ISOLATED FROM PERIPHERAL BLOOD

PMN store elastase in the cytoplasmic azurophilic granules and upon activation, release the enzyme in an active form as a part of the response to an acute infection or injury (43, 44). Targeted proteins besides elastin include plasminogen, collagen type IV, fibronectin, proteoglycans, coagulation factors, immunoglobulins, and complement components. Thus, PMN elastase with its broad specificity is eminently suited to clear the debris attending inflammation. In the context of this role in inflammation, of particular interest is the recent observation that PMN can cleave apo[a] and that this cleavage occurs specifically via the released elastase en-

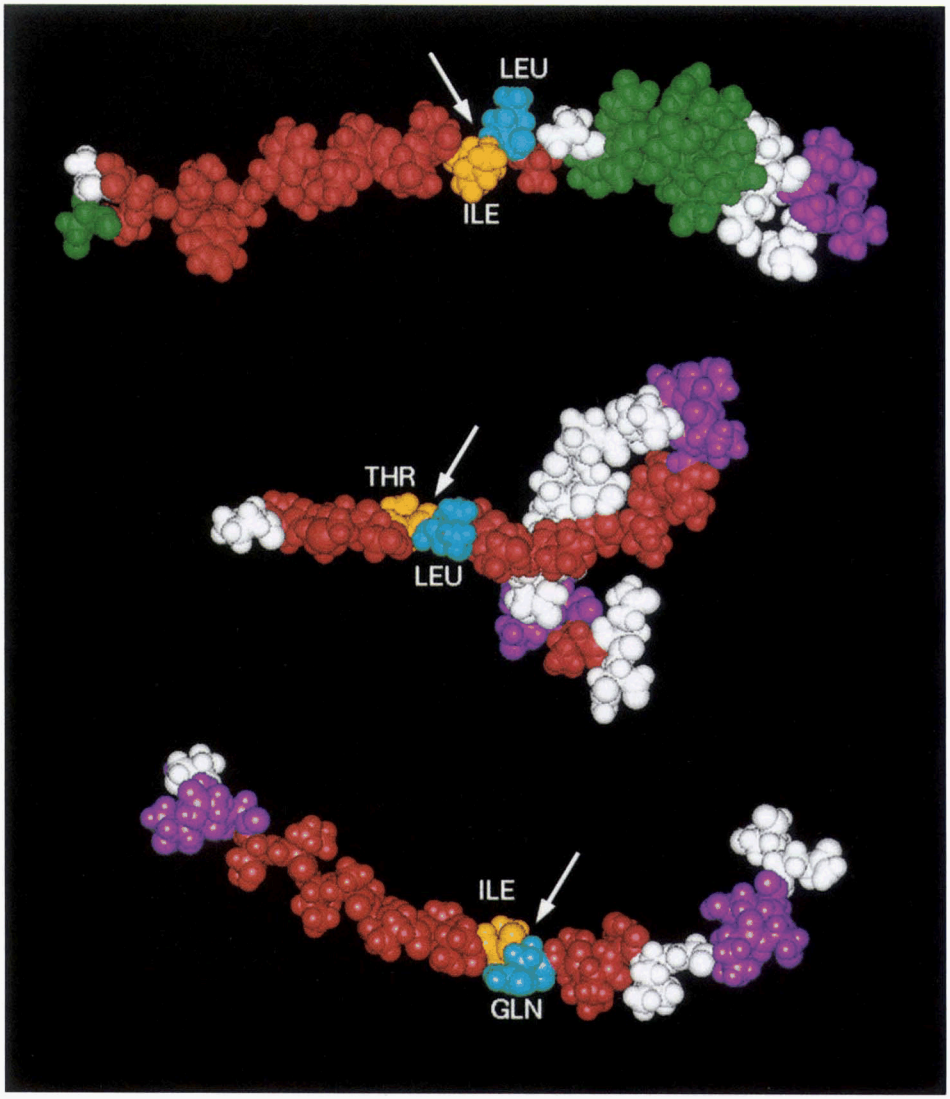


Fig. 5. Computer-simulated models of the linkers containing the peptide bonds cleaved by PMN elastase. The cut sites are marked by an arrow. The α-helical domains are in green, the β-strand is in red, the turns are in purple, and white represents the random conformation. Top, linker 4 which connects KIV-4 to KIV-5; middle, linker 7 which connects KIV-7 to KIV-8; bottom, linker 10 which connects KIV-10 to KV. Modeling was performed on a molecular graphics workstation from Silicon Graphics Inc. using the modeling system Insight II v.95.0 and the programs Builder, Biopolymer and Discover (Biosym/MSI, San Diego, CA). As crystallographic coordinates are not available for the linker regions we used the amino acid sequence deduced from the cDNA sequence and built each amino acid sequentially in a linear fashion. The secondary structure was then constructed based on the algorithms of Chou-Fasman (90) and Garnier/Robeson (91) and the model was subjected to energy minimization.

zyme (37). Of note, PMN cleaves apo[a] at the same sites as purified leukocyte elastase. Models of the three linkers containing the elastase cut sites are shown in **Fig. 5.** It is of interest to note that the cleavage site occurs in a predicted beta-strand region. The same cleavage pattern of apo[a] is obtained when PMN is incubated with Lp[a] indicating that the disulfide linkage between apo[a] and apoB-100 is not a hindrance to the elastase action. As a consequence, the truncated apo[a] which contains kringle IV-9, the site of the unpaired cysteine involved in inter-disulfide linkage, generates miniLp[a] particles. Based on electrophoretic, ultracentrifugal criteria, and amino acid sequence analysis, these particles exhibit the properties of those formed by the action of purified leukocyte elastase on Lp[a]. These observations suggest that elastase-mediated proteolytic events can occur in vivo as discussed in the following section.

POTENTIAL SIGNIFICANCE OF PMN ELASTASE IN LP[a] BIOLOGY

Apo[a] fragments are spontaneously present in the plasma and urine of human subjects (10–12, 36) as well as cynamolgus monkeys (10). In human subjects, the urine concentrations are about 1% of the total pool of circulating apo[a] (45); moreover, a correlation between plasma concentrations of Lp[a] and urine apo[a] fragments has been observed (12). Similarly, the reduction of the plasma Lp[a] levels by plasmapheresis results in a parallel decrease of the urinary fragments (46). Of note, the urinary fragments derive mainly from the NH_2-terminal domain of apo[a] (12, 36), the very F1 domain that is generated in vitro by the action of PMN elastase. Moreover, the same type of fragments are observed in the urine of mice injected intravenously with either apo[a] or F1 (36). Thus, it is apparent that through an elastase-dependent enzymatic action, apo[a] can be post-translationally degraded into units, some of which appear to be targeted for urinary excretion and some for binding to apoB-100, fibrinogen, and fibronectin. It is also important to note that all of the elastase cleavages are in the linker regions of apo[a] and that the one occurring between K IV-4 and IV-5 effects a critical separation between two domains, one functional, F2, and the other, F1, with no apparent function, based on the parameters studied thus far, namely binding to lysine, fibrinogen, and fibronectin (36). Under physiological conditions, the in vivo fragmentation of Lp[a]/apo[a] appears to be a relatively modest event from the quantitative standpoint. However, in inflammatory states and particularly at tissue sites where the elastase activity may be unopposed by elastase inhibitors, apo[a] fragmentation could be a relatively large event.

An interesting outcome of the elastase studies is miniLp[a], a particle having a truncated apo[a] containing only the functional domain, F2, and not the multiple K IV-2 repeats. The biological significance of miniLp[a] is unclear at this time. However, data from this laboratory have shown that miniLp[a], obtained by PMN elastase digestion, binds fibrinogen and fibronectin more avidly than undigested Lp[a] (37), suggesting that the N-terminal domain, as a component of whole apo[a], may have some inhibitory effect on the function of the COOH-terminal domain. Moreover, upon intravenous injection into the mouse, miniLp[a] resides in the plasma longer than Lp[a] (36). This observation invites the speculation that differences might exist in the rate of endothelial migration between these two particles and/or avidity for high affinity sites on the cell membrane, probably influenced by the presence or absence of the N-terminal domain. The notion that elastase enzymes are capable of modulating the catabolic fate and function of Lp[a] opens interesting new vistas on the mechanisms of cardiovascular pathogenicity of this lipoprotein as discussed below.

POTENTIAL ROLE OF APO[a] FRAGMENTS IN THE CARDIOVASCULAR PATHOGENICITY OF LP[a]

PMN have been reported to play a role in reperfusion injury after myocardial infarction (47). In terms of the atherosclerotic process, activated PMN are present only in a small number in the affected intima (48). On the other hand, Kling, Holzschuh, and Betz (49) have recently found that in the early phase of atherosclerosis of rabbits fed a high cholesterol diet, PMN were the most abundant cells in the intima and were later replaced by monocytes and lymphocytes. Of interest, in a 10-year cooperative study involving 4,860 British men living in South Wales and the West of England, the total leukocyte count, mainly represented by neutrophils, predicted ischemic heart disease after adjusting for the classical risk factors (50). Moreover, in support of the notion that inflammation is an important feature of the atherosclerotic lesion, the results of recent 2-year studies conducted on 2,121 outpatients with stable and unstable angina showed that raised concentrations of the C-reactive protein are predictors of coronary events (51). Similarly, elevated baseline plasma levels of C-reactive protein were found in 543 apparently healthy men participating in the Physician's Health Study to predict the risk for future myocardial infarction and stroke (52). It is also important to note that several studies have shown a significant association between coronary heart disease and microbial infection both within and outside the arterial lesions (53–59). Thus, in the framework of an acute phase reaction, apo[a] fragmentation may occur via PMN activation. Of note, increased plasma levels of Lp[a] have been reported in relation to an acute phase reaction (60–62); however, we have previously speculated that such an increase may not be real but related to changes in epitope expression and, thus, antibody reactivity secondary to apo[a] fragmentation (36). In considering atherosclerosis as a chronic inflammatory process, it should be noted that the main cellular components of the diseased intima are represented by activated macrophages and smooth muscle cells, both capable of secreting elastases along with type IV collagenase or matrix metalloproteinse 2, a metalloenzyme which, upon activation, is able to cleave apo[a] (63). In this regard, we have recently shown that the human monocytoid cell line U-937 secretes into the me-

dium a proteolytic activity that cleaves Lp[a] and apo[a] and, like PMN elastase, is inhibited by methoxy-succinyl-Ala-Ala-Pro-Val-CH_2Cl, a specific elastase inhibitor (38). This finding is anticipated by the notion that PMN elastase is synthesized at the level of the promyelocytic cells and is transmitted in the active form to either the PMN lineage or to the monocytoid cell line. By immunological criteria, Lp[a] has been shown to accumulate in the subendothelial matrix of arteries with atherosclerotic lesions and also in saphenous venous grafts after coronary bypass surgery (64–66). However, it is not known whether the immunoreactive Lp[a] is intact or represents fragments from either enzymatic or non-enzymatic oxidative derivation. Fragments of apo[a] have been reported in the atherosclerotic plaque (13), and it is tempting to speculate that they may have originated from elastase-dependent proteolytic events. As already discussed, the inflammatory milieu of the intima of a diseased artery can favor the presence of enzymes of the elastase and metalloproteinase family, likely causing the disruption of the structural integrity of the Lp[a] particle. Fragmentation attending the action of these proteolytic enzymes would generate miniLp[a] and apo[a] fragments, probably of the F2 type, as the F1 fragments would readily diffuse from the sub-endothelial matrix, return to the circulation, and be eliminated either in the urine or other clearance mechanisms. The interaction between miniLp[a] and either proteoglycans or glycosaminoglycans would generate large macromolecular complexes with an atherogenic potential due to their targeting for uptake by activated intimal macrophages leading to foam cell formation. In turn, the apo[a] fragments not linked to apoB-100 would not be competent to generate foam cells and be instead thrombogenic through binding to fibrin. According to results obtained in this laboratory (37), an increased elastase activity can also cause a partial proteolysis of LDL whether this lipoprotein particle is authentic or a member of Lp[a]. Moreover, elastase-modified LDL has been shown to have an atherogeniic potential when incubated with human monocyte-derived macrophages (67) and to also bind more avidly than unmodified LDL to proteoglycans (O. Klezovitch and A. M. Scanu, unpublished observations).

Lipid peroxidation has gained widespread acceptance as a major determinant in the changes in surface charge, particle destabilization, and protein fragmentation of apoB-100-containing lipoproteins in atherogenesis (68, 69). We propose that enzymatic processes may contribute to, and augment, these biochemical changes and that the proportionate contributions may depend upon tissue milieu, extent of lipid peroxidation, and/or degree of inflammation. Elastase-dependent changes in Lp[a]/apo[a] and apoB-100 may occur early at sites of vascular injury and the resulting products may become relatively more susceptible to oxidative modifications. In order to prove this hypothesis, we need to have access to specific antibodies able to differentiate products deriving from either enzymatic or non-enzymatic events and to techniques for the isolation of these products on a sufficiently large scale to perrmit characterization.

In terms of pathogenetic mechanisms, the better recognized current concept is that based on the homology between apo[a] and plasminogen. According to this concept, Lp[a] would interfere with the process of plasmin generation (70, 71) and as a consequence lead to a decreased fibrinolysis and an increased accumulation of fibrin in the atherosclerotic plaque. This concept would not rule out an elastase-mediated accumulation of fibrin at lesion sites except that in the latter case, fragments rather than intact apo[a] would be interacting with the fibrin deposited in the atherosclerotic plaque. At present there is no clinical evidence supporting an association between high plasma levels of Lp[a] and a fibrinolytic defect short of the case of transgenic mice expressing high plasma levels of human apo[a], shown to exhibit a delayed lysis time of thrombi artificially produced in the carotid artery of those animals (72). An additional, rather attractive hypothesis is that by Grainger et al. (73, 74) who have provided experimental evidence that a diminished plasmin generation can cause a decreased formation of an active TGF-β, resulting in an uninhibited increase in smooth muscle cell proliferation. However, the notion of a TGF-β as an inhibitor of smooth muscle cell migration is not universally accepted as such an effect varies depending on the experimental in vitro conditions (75–77). From the above it is apparent that the complexities and uncertainties relating to the pathogenesis of the atherosclerotic process preclude at this time the identification of specific mechanisms for the role of cardiovascular pathogenicity of Lp[a]. In this conceptual framework, this pathogenicity is likely to be the result of an interplay of factors comprising the intrinsic properties of Lp[a], the inflammatory status of the arterial wall, the relative abundance and function of the extracellular matrix, the activity of the cellular components of the intima and of the enzymes with a potential for cleaving Lp[a]/apo[a].

CONCLUSIONS AND FUTURE DIRECTIONS

Apo[a], which has been difficult to study because of its large size and its covalent attachment to apoB-100 in Lp[a], is now shown to be amenable to enzymatic

dissection by biological enzymes of the serine protease family and particularly elastases. This enzymatic dissection provides a powerful tool for studying individual apo[a] domains and to correlate their structural properties to function, particularly in terms of binding to macrolecules relevant to the athero-thrombotic process. Besides PMN, activated macrophages and smooth muscle cells, major cellular components of a diseased arterial intima, can secrete elastase and a metalloproteinase also able to cleave apo[a] (63) suggesting that fragmentation of Lp[a] and apo[a] is one of the manifestations of the atherosclerotic process. Apo[a] immunoreactive fragments have been reported in the human atherosclerotic plaque and assumed to be secondary to oxidative events (13). This hypothesis, although attractive, lacks experimental evidence and does not exclude alternative or additional mechanisms responsible for Lp[a]/apo[a] fragmentation. The in vivo participation of enzymes of the serine protease family in the modulation of Lp[a]/apo[a] catabolism deserves attention because it introduces novel pathogenetic insights into the cardiovascular pathogenicity of Lp[a] and calls for more research into unsuspected role(s) that specific apo[a] linkers may play in this catabolism. The view that Lp[a] fragmentation may be part of an inflammatory process of the arterial vessel also suggests novel therapeutic modalities directed at developing specific elastase inhibitors capable of operating at the level of the arterial intima. In this context, elafin, an elastase inhibitor, has been recently shown to attenuate coronary arteriopathy and reduce myocardial necrosis in rabbits after heterotopic cardiac transplantation (78). However, the safety and applicability of this agent in human subjects still remains to be established.

The possibility of a ready access to kringle/linker domains that can be easily obtained from Lp[a] isolated from human plasma also provides an important experimental basis for research activities aimed at filling the many existing gaps of knowledge surrounding Lp[a] and its apo[a] derivatives. Because a structural integrity is required for an appropriate kringle function, information is needed on the factors controlling folding and an initial study to this effect has just appeared (79). We also need to define the carbohydrate content and composition of each apo[a] domain, the reason for the abundance of O-linked carbohydrates in the linker regions and the overall role(s) of carbohydrates in apo[a] function. Also in need of definition are the sites responsible for the binding of apo[a] to structures relevant to the atherosclerotic process (i.e., fibronectin, collagen, proteoglycans and glycosaminoglycans, etc.) or the thrombotic process (fibrin(ogen)). This information should also aid in the investigation of the molecular basis for the interindividual polymorphism in the lysine (80, 81) and fibrinogen (82–84) binding properties of Lp[a] recently shown to occur in human subjects irrespective of gender, age, and plasma Lp[a] levels. In terms of apo[a] size isoforms, their potential role in the cardiovascular pathogenicity of Lp[a] is still controversial. Sandholzer et al. (85) have reported that small apo[a] isoforms are more frequent in patients with coronary heart disease than in controls. However, this association was not observed by Farrer et al. (86). In a prospective case control study, Wild, Fortmann, and Marcovina (87) have shown that apo[a] size represents a risk for coronary heart disease in men, but not in women. More recently, Kraft et al. (88) analyzed the apo[a] gene of selected Tyrolean patients and found a significant negative correlation between number of kringle IV repeats and risk for coronary heart disease. This observation, if corroborated by larger scale studies, would invite questions of a mechanistic nature. As small size isoforms are associated with high plasma levels of Lp[a], they would localize in the arterial wall in preference to the larger isoforms. In such a case, any free apo[a] would be mainly represented by F2-like fragments which we are speculating to have a high atherothrombogenic potential. Accordingly, the low atherothrombogenic potential of the larger apo[a] isoforms having a high number of kringle IV-2 repeats, would be attributable, at least in part, to their inability to generate plasma levels of Lp[a] sufficient to cause an abnormal accumulation of this lipoprotein and/or derivatives in the vessel wall. Much of the current information on the cardiovascular pathogenicity of Lp[a] has been based on plasma Lp[a] levels and, in some cases, divergent results have been reported (2, 4). The notion that apo[a] is made of domains that may vary in function among human subjects invites attention to the fact that conclusions on the cardiovascular pathogenicity of Lp[a] should be based not only on plasma concentrations but should also include an assessment of the functional properties of this lipoprotein particle. Moreover, we should define the origin of the apo[a] fragments spontaneously present in the plasma and urine of human subjects. Under physiological conditions, the production in the plasma of these fragments by a PMN elastase-mediated mechanism would be rather modest because of the presence of powerful natural inhibitors. On the other hand, in acute inflammatory states associated with an increased PMN activation unmatched by specific inhibitors, the levels of apo[a] fragments in the plasma and the urine would rise. In such a case, some of these fragments may originate at a tissue level and cycle back to the circulation by a reverse migration through the cell membrane. In turn, from the atherothrombogenic standpoint, proteolysis of Lp[a]/apo[a] in the arterial intima would lead to the retention of F2

and/or miniLp[a] via their high affinity for the members of the extracellular matrix.

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