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Morphological detection and quantification of lipoprotein(a) deposition in atheromatous lesions of human aorta and coronary arteries

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Summary. Lipoprotein(a), as an atherogenic particle, represents an independent risk factor for coronary heart disease. In the present study the morphological distribution of apoprotein (a) and apoprotein B within the arterial wall is described. Apoprotein B, a constituent of very low-density lipoprotein, low-density lipoprotein and lipoprotein(a) has previously been demonstrated in atheromatous lesions. Lipoprotein(a) possesses an additional protein, designated apoprotein (a). Autopsy material ($n=74$) from the left coronary artery and from the thoracic aorta has been examined by means of immunohistochemistry and both apoprotein (a) and apoprotein B were detected, primarily associated with the extracellular matrix and accumulating in lesions in the arterial wall. The staining pattern for both antigens was almost always found to be congruent, suggesting that the detection of (a)-antigen has to be attributed at least in part to the presence of lipoprotein(a). It is concluded that both low-density lipoprotein and lipoprotein(a) have an important role in the pathogenesis of atherosclerosis.

Key words: Arteriosclerosis – Lipoprotein(a) – Apoprotein (a) – Immunohistochemistry

Introduction

Lipoprotein(a) [Lp(a)] is a lipoprotein that closely resembles low-density lipoprotein (LDL). Both lipoproteins have a similar lipid composition and both contain apoprotein B (apoB). In addition, Lp(a) consists of a protein called apoprotein (a) [apo(a)], which is attached to the apoB by disulphide bonds (Utermann et al. 1969, 1987; Simons et al. 1970; Ehnholm et al. 1972). Lp(a) was first described by Blumberg et al. (1962) and Berg (1963). The molecular weight of apo(a) is higher than 500 kDa (Utermann et al. 1987). Lp(a) floats in a density range of 1.05–1.12 g/ml. It binds to the LDL receptor with lower affinity than LDL (Krempler et al. 1984; Maartmann and Berg 1981; Havekes et al. 1981; Floren et al. 1981; Armstrong et al. 1985). Its clearance from the plasma has not yet been fully evaluated. Lp(a) is synthesized in the liver and is probably secreted by hepatocytes, associated with apoB.

The pathophysiological relevance of Lp(a) is evident in the positive correlation between elevated serum levels of Lp(a) and coronary heart disease shown in a number of epidemiological studies (Wottawa et al. 1984; Schriewer et al. 1984; Dahlen et al. 1986; Armstrong et al. 1986). Approximately 30% of a normal population express atherogenic serum levels of Lp(a) that are above 25 mg/dl; 40% of patients suffering from coronary heart disease express elevated Lp(a) serum levels.

In previous morphological studies apoB has been detected in the arterial wall by means of immunohistochemistry (Hoff et al. 1977, 1978; Hoff and Bond 1983; Yomantas et al. 1984; Yamauchi and Hoff 1984; Carter et al. 1987). From these investigations it has been deduced that either LDL and/or very low-density lipoprotein (VLDL) are present. Only one study (Walton et al. 1974) has described apo(a) in the arterial wall. Interestingly, in that report it was concluded that Lp(a) does not participate in atherogenesis.

Against a background of increasing epidemiological evidence about the atherogenic properties of Lp(a) and with the availability of an apo(a)-specific monoclonal antibody, the present study investigates whether or not there exists a correlation between immunoreactivity of apo(a) and apoB in the arterial wall. A positive finding indicates that the localization of apoB has to be related, at least in part, to the presence of Lp(a). Autopsy tissue from the thoracic aorta and the left coronary artery has been examined by means of immunohistochemistry (Hsu et al. 1981; Niendorf et al. 1987) in combination with morphometric analysis in order to determine whether

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Lp(a) can be detected preferentially in atheromatous lesions of the arterial wall.

Materials and methods

Autopsy tissue ($n=74$) from patients who died at the age of 0–98 years (39 females, 35 males, approximately 10 samples representing each decade) was obtained from the Institutes of Pathology and Forensic Medicine at the University of Hamburg and was taken between 24 and 47 h post mortem. The slices were cut from the thoracic aorta in the region of the first intercostal arteries and from the first 2 cm of the left coronary artery. From atherosclerotic vessels two samples were taken: one from an atheromatous area and a second, if possible, from an adjacent area which was grossly normal. The tissue was formalin-fixed [3.7% buffered in phosphate-buffered saline (PBS)] and paraffin-embedded by standard procedures. Haematoxylin and eosin and elastic-van-Gieson staining were performed by routine methods.

Polyclonal goat anti-apoB (Immuno, Vienna) was applied to the tissue sections in a 1:1000 dilution. Monoclonal mouse-anti(a) has been produced in the laboratory of one of the authors (U. Beisiegel) and will be described and characterised elsewhere (paper in preparation). This antibody has been checked for a possible cross-reactivity with plasminogen, which was excluded.

For immunohistochemistry deparaffinised 2-µm sections were mounted on Zementit-coated slides. They were allowed to dry at 60° C overnight. Endogenous peroxidase was inhibited by a 10-min treatment with methanol-H_2O_2 (174 ml methanol + 6 ml 30% H_2O_2). Thereafter tissue sections were washed for 10 min in PBS and pre-incubated with normal horse serum [apo(a)] or normal-rabbit serum (apoB) for 30 min at room temperature. The serum was decanted and the specific first antibody [monoclonal anti-apo(a) or polyclonal anti-apoB] was applied for 24 h at 6° C. Incubation with the primary antibody was followed by three washes in PBS for 10 min each. After that the sections were incubated with biotinylated secondary antibodies [horse anti-mouse for Lp(a) detection and rabbit anti-goat for apoB] which were both diluted 1:200 in PBS. This was again followed by three washes of 10 min each. Thereafter the sections were incubated for 30 min with the avidin-biotin reagents (Vector Laboratories, Burlingame, Calif.) and washed again three times for 10 min. Sections were then incubated with diaminobenzidine as a chromogenic substrate for 6 min. They were rinsed for 5 min in plain water and counter-stained with haemalaum for 5 min, dehydrated in alcohol and mounted in Eukit.

In control incubations non-immune serum of the same species in which the specific first antibody had been raised (normal mouse serum or normal goat) was applied instead of the first antibody.

Sections of the arterial wall were classified as "normal" (no lesion and intimal thickening), "fatty streak" (accumulation of foam cells), "fibrous plaque" (smooth muscle cell and collagen accumulation) and "complicated lesion" (any fibrous lesion including in addition either necrosis, haemorrhage, thrombosis or calcification).

Immunoreactivity within the arterial wall was estimated by semiquantitative methods and a staining score was deduced from the following formula:

$$SC = (\%SA_{LA}/100) \times (I_{SA}) \times (\%LA_{TSA}/100),$$

(where SC = staining score; SA_{LA} = stained area with regard to lesional area; I_{SA} = intensity of stained area; LA_{TSA} = lesion area with regard to total section area).

This included the area and intensity of staining in a given tissue section. A value ranging from 0 to 12 thus resulted from the product of intensity (0–12) and the percentage of a stained area (divided by 100) after consideration of the percentage of lesional area within the given total section through the arterial wall. Thus the highest possible staining score was 12. The morphometric analysis was performed by two independent investigators.

Results

The distribution of staining for apo(a) and apoB was found to be located almost exclusively in the intima of the arterial wall in both thoracic aorta and left coronary artery. The media expressed staining for either antigen in general. In 5% of the total sample number a restricted immunoreactivity at the cellular level was observed in smooth muscle cells of the media. The lumina of adventitial vessles (vasa vasora) showed staining for both antigens. The adventitia itself expressed a low level of diffuse staining in some cases.

The deposition pattern of both antigens in the normal intima is summarized schematically in Fig. 1 A–G. In non-lesional areas there was either no staining at all (Fig. 2), or fine spot-like or a fine striped staining. Both patterns were found in the near luminal part and the near media part (Fig. 3) or were distributed over the whole area of the intima. In no case was a marked aggregation of staining observed in non-lesional areas. In contrast, in fibrous plaques and complicated lesions dense bundle-like staining pattern was seen, for both antigens. Predominant localization of both antigens within fibrous caps was seen this either reached to the luminal border of the intima (Fig. 1; CL and FP type A) or covered the necrotic core of a complicated lesion or was found at the edges of necrotic cores (Fig. 5). The distribution patterns of apo(a) and apoB in lesional areas are summarized in Fig. 1 H–L.

At the cellular level predominant localization of apo(a) and apoB in the extracellular matrix has been observed.

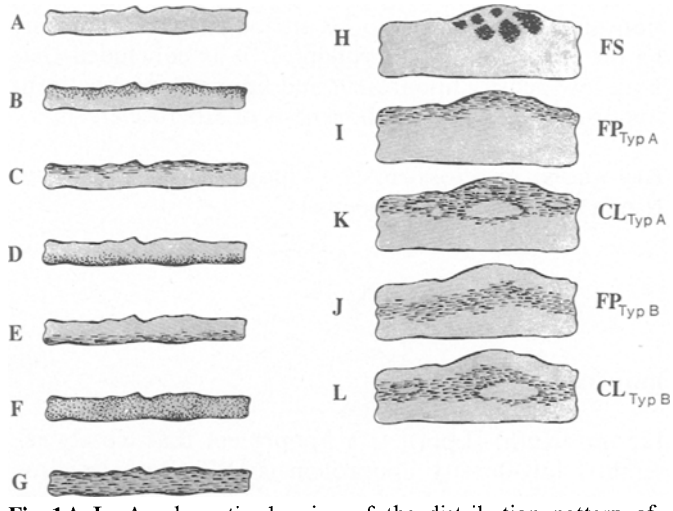


Fig. 1 A–L. A schematic drawing of the distribution pattern of apo(a) and apoB deposition. **A–G** Normal intima (i.e. non-lesional areas) of the arterial wall. **H–L** Different types of lesions (*FS*, fatty streak; *FP*, fibrous plaque; *CL*, complicated lesion). Type A and B of FP and CL differ from each other in as much as the staining reaches the luminal border of the intima (type A) or is separated from the luminal border by a small margin. Only the lamina intima is demonstrated in **A–L**

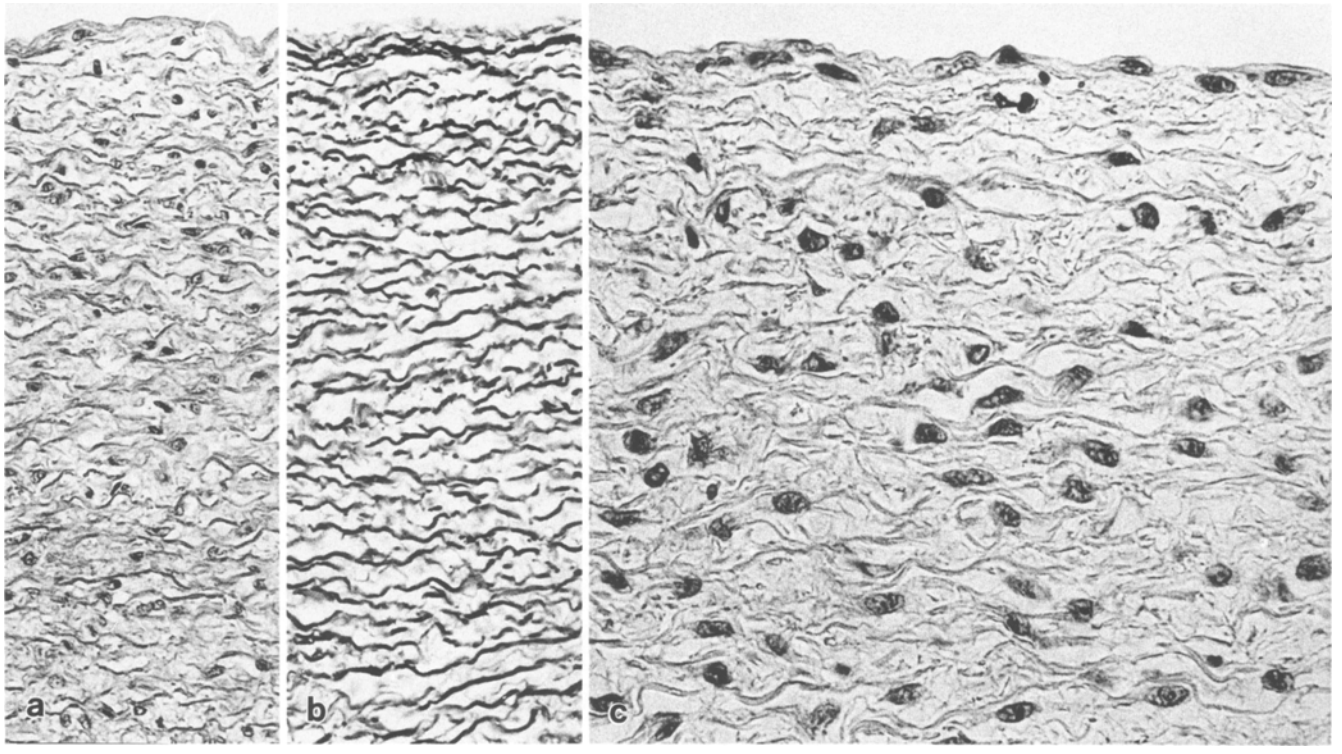


Fig. 2a–c. All taken from samples that did not expose plaque formation. The aorta of a newborn with no visible lesion (H & E stain). **b** The same section as **a** but stained by elastic van Gieson. **c** Immunohistochemistry for apo(a) in tissue of the same subject as shown in **a** and **b**. No staining can be observed. **a–c** ×250

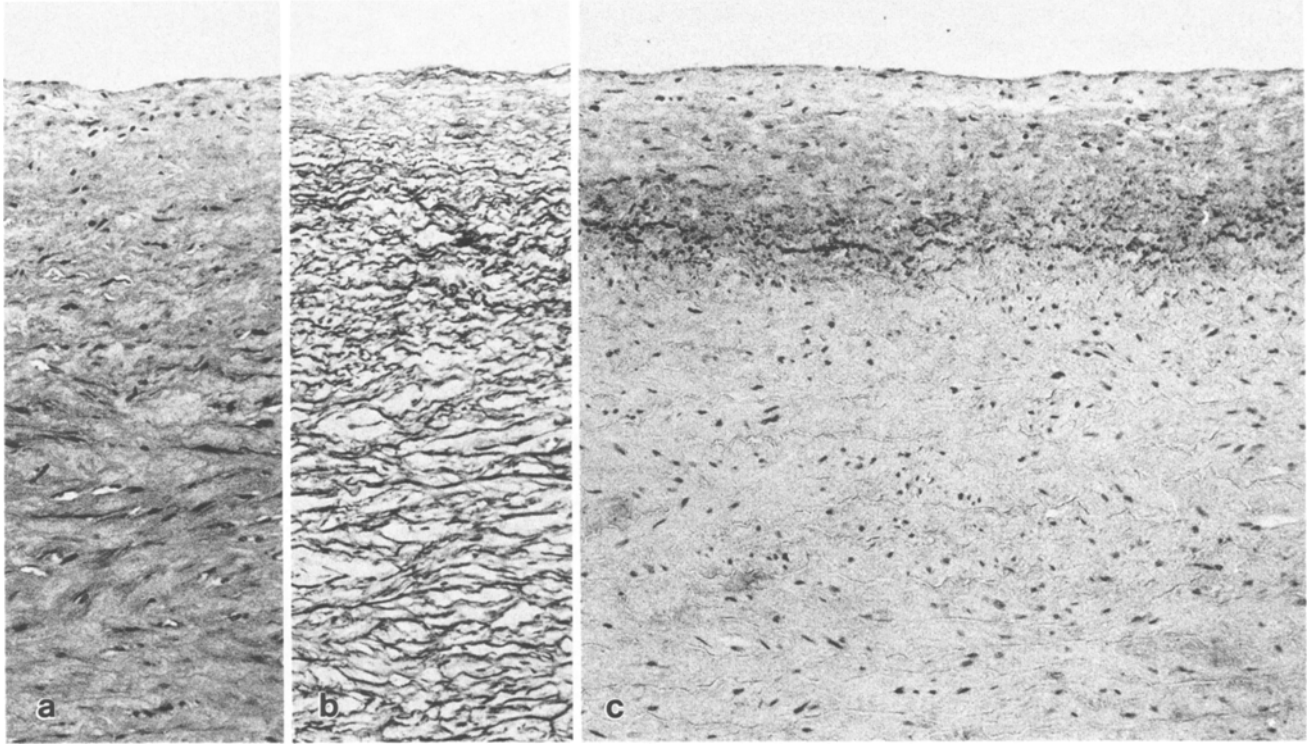
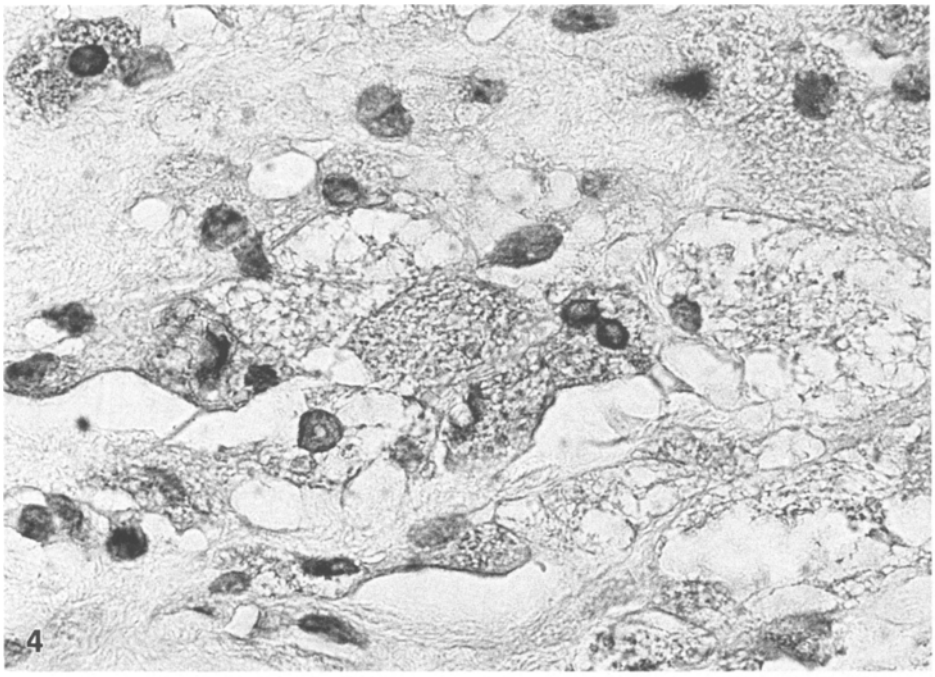


Fig. 3a, b. The aorta of a 55-year-old male with moderate intimal thickening. **a** H & E stain; **b** elastic van Gieson. **c** Immunocytochemical demonstration of apo(a) in a fine spot-like pattern that is mainly located in the extracellular space and restricted to the area of the intima. Immunocytochemical staining accumulates at the borderline between intima and media. **a–c** ×100



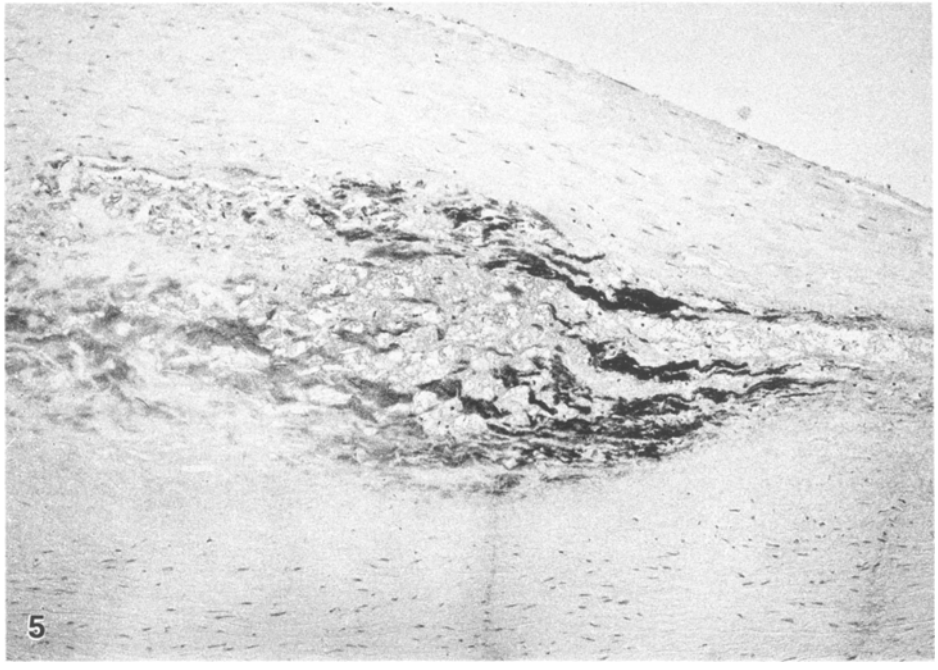


Fig. 4. Aorta of a 45-year-old male with a fibrofatty plaque which is composed of fibrous masses and of foam cells. Detection of (a) antigen exhibits an intracellular (foam cell) staining. ×1000

Fig. 5. Apo(a) immunostaining in a complicated lesion of the aorta in a 76-year-old subject. Extracellular staining can be seen in the fibrous cap and at the edges of this lesion whereas the necrotic core shows only faint staining

Exceptionally, some foam cells within fatty streaks or complicated lesions (Fig. 4) and a few smooth muscle cells of the media contain certain amounts of both antigens. In summary apo(a) and apoB are located extracellularly almost exclusively.

Comparison of the distribution pattern of both antigens with regard to localization and intensity exhibits a high degree of congruency for apo (a)- and apoB staining in all lesion areas. Localization of apo(a) and apoB is shown to be present in atheromatous lesions of the aorta as well as in the coronary artery as shown in Figs. 2–5 [apo(a) localization]. The co-localization of both proteins is demonstrated in Figs. 6 and 7.

Quantification of immunoreactivity shows a significantly more intensive staining for both antigens in regions of the intima that show either fatty streaks, fibrous plaques or complicated lesions than in areas of normal intima. Plaque area as a total has a staining score (mean value for both lipoproteins in aorta and coronary artery) of 6.5 compared with 2.5 in non-lesional areas. The most pronounced aggregation of both proteins is observed in the coronary artery exhibiting a staining score of 5.1 for apo(a) and 6.7 for apoB in lesional areas compared with 1.2 [apo (a)] and 1.8 (apoB) in non-lesional areas. All values for the immunoreactivity are listed in Table 1.

Discussion

Increasing numbers of epidemiological studies (Wottawa et al. 1984; Schriewer et al. 1984; Dahlen et al. 1986; Armstrong et al. 1986) have indicated the value of investigating the distribution of apo(a) and apoB in a larger

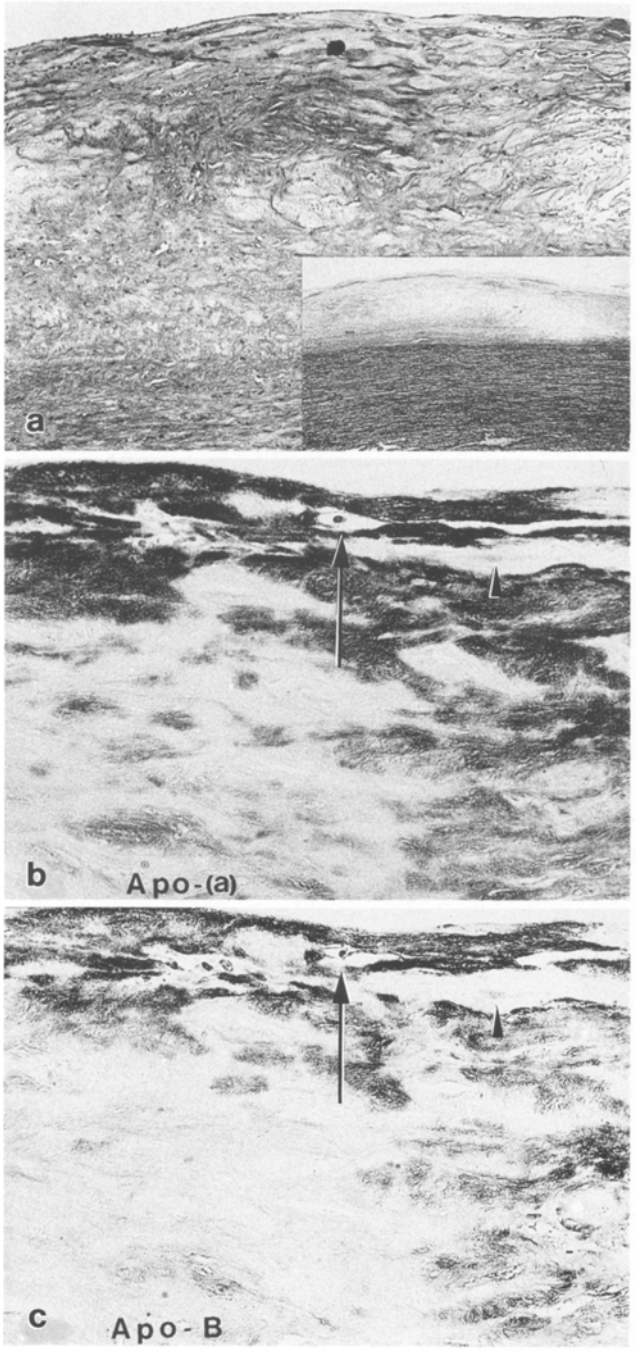


Fig. 6a–c. A complicated lesion of the aorta of a 77-year-old female. **a** Haematoxylin and eosin, ×100. The *inset* demonstrates the same section (elastic van Gieson, ×25). **b, c** The same area as demonstrated in **a** with staining for apo(a) (**b**) and apoB (**c**). The fibrous cap of the lesion is stained in a striped manner. The necrotic core shows a slight diffuse staining. Cellular components of this lesion are excluded from staining. Furthermore the strong congruency of both adjacent sections can be revealed. Corresponding cells are marked by *arrows* and *arrowheads* in the corresponding pictures (**b, c**)

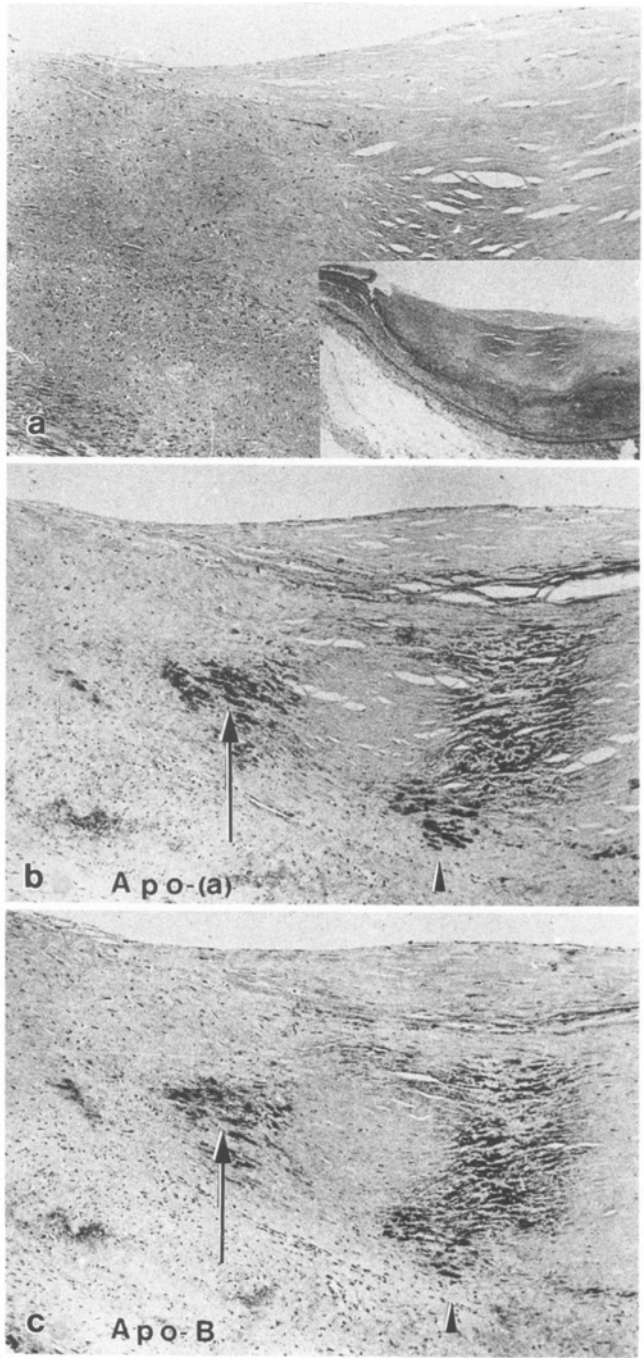


Fig. 7a–c. A section through a complicated lesion of the coronary artery of a 77-year-old female (same subject as shown in Fig. 6, ×100). **a** Haematoxylin and eosin; the *inset* demonstrates an overview of this section (elastic van Gieson, ×25). **b, c** The same area as demonstrated in **a** with staining for apo(a) (**b**) and apoB (**c**). Again cellular components of this lesion are excluded from staining. A strong congruency of apoprotein staining in both adjacent sections can be revealed. Corresponding areas are marked by *arrows* and *arrowheads* in the corresponding pictures (**b, c**)

number of cases. The intriguing immunological problem that results from a close similarity between plasminogen and apo(a) (Havekes et al. 1981; Floren et al. 1981) was resolved by the availability of a monoclonal antibody that detects the apo(a) and does not cross-react. The results from this study reveal a staining pattern for apo(a) and apoB located in the intima of the arterial wall with a preference for lesions is predominantly in the extracellular space, and is congruent in adjacent sections.

Determination of staining intensity in lesions and non-lesional areas clearly demonstrates that apo(a) and apoB are enriched in lesions. This finding supports the epidemiological observation that Lp(a) is associated

Table 1. Morphometric analysis of apoprotein distribution in the arterial wall

Normal ($n = 49$)	FS ($n = 62$)	FP ($n = 45$)	CL ($n = 36$)
Aorta: Apo B			
4.2 ±3.1	7.9 ±3.7	7.6 ±3.0	9.0 ±1.8
$p < 0.001$		NS	
$p < 0.001$			$p < 0.02$
	NS		
$p < 0.001$			
Aorta: Apo(a)			
3.1 ±2.1	6.5 ±2.8	6.4 ±2.8	7.1 ±1.5
$p < 0.001$		NS	
$p < 0.001$			NS
	NS		
$p < 0.001$			

Normal ($n = 46$)	FS ($n = 46$)	FP ($n = 52$)	CL ($n = 48$)
Coronary Artery: Apo B			
1.8 ±1.9	5.8 ±3.3	4.8 ±3.1	7.6 ±2.6
$p < 0.001$		$p < 0.01$	
$p < 0.001$			$p < 0.001$
	NS		
$p < 0.001$			
Coronary Artery: Apo(a)			
1.2 ±3.1	4.9 ±3.4	4.1 ±3.0	6.4 ±2.8
$p < 0.001$		$p < 0.05$	
$p < 0.001$			$p < 0.001$
	NS		
$p < 0.001$			

Table 1. Relative scores for apo(a)- and B deposition in human coronary and aorta tissue sections as determined by morphometric analysis (for details see Materials and methods)
FS, Fatty streak; FP, fibrous plaque; CL, complicated lesion. Significance was calculated using Student's *t*-test

with the appearance of atherosclerosis. Both antigens show a comparable tissue distribution in the thoracic aorta and the coronary artery. Since the aorta exhibits fatty streaks that are composed of foam cells more frequently than the coronary artery, with its rather fibrous or complicated lesions, it is noteworthy that the number of intracellularly stained cells is higher in the aorta than in the coronary vessel. Under the premise that intimal thickening can be considered to be a physiological age-related process it is important to note that deposition of both proteins occurs in parts of the intima not affected by morphologically detectable lesions. However, this is observed to a much lesser extent than in lesions of the arterial wall. The demonstration of protein deposition in non-lesional areas might be interpreted as the detection of earliest stages of the development of atheromatous lesions.

The mechanism of accumulation of lipoproteins in the arterial wall is still under debate (Kratzin et al. 1972; Smith et al. 1976; Hollander et al. 1979; Hoff et al. 1979). LDL is suspected to be modified and enriched in the intima that has undergone a primary injury at the site of the endothelial barrier. Concerning the Lp(a) particle two hypotheses are under discussion: Lp(a) as a lipoprotein might diffuse into the intima and either stick to the intercellular components such as glycosaminoglycans or be directly taken up by monocyte-derived macrophages or smooth muscle cells. Since Lp(a) is known to bind to glycosaminoglycans with a higher affinity than does LDL (Smith et al. 1976; Hollander et al. 1979) this particle is predisposed to be entrapped in the intercellular matrix. Subsequently it might be phagocytosed, in the sense of a repair or clearance mechanism and phagocytosis and receptor-mediated endocytosis lead to lysosomal degradation of the incorporated ligand. One would not expect ligands which have been undergone lysosomal degradation to be detectable by immunological methods and it seems unlikely that we are detecting apoproteins that have undergone phagocytosis.

We therefore favour a second hypothesis to explain Lp(a) accumulation in the arterial wall, based on the similarity of this lipoprotein with plasminogen. The apo(a) molecule is made up predominantly of kringle structures having a high degree of homology with plasminogen (Eaton et al. 1987; McLean et al. 1987). Lp(a) might therefore bind to sites where fibrinogen is polymerized. In this regard Lp(a) might play a fatal role interfering with fibrinolysis, which has to occur in a regular manner to prevent fibrotic organization of mural thrombi triggering the formation of complicated lesions. Preliminary results from our group confirm speculations about a Lp(a)-fibrin association. Morphologically a striking congruency of fibrinogen and apo(a) was observed in a limited number of cases (data not shown). The distribution patterns of both proteins with regard to the intra- and extracellular localization also favour a mechanism where Lp(a) either sticks to extracellular matrix components or is entrapped in thrombi, due to its similarity with plasminogen. Biochemical data (Rath et al. 1989) reveal a considerable amount of gradient-extracted apo(a) associated with intact lipoprotein particles. This finding corroborates the concept of a predominantly extracellular deposition. In this work we find apo(a) and apoB to be deposited mainly extracellularly, although weak intracellular staining within foam cells can be observed occasionally.

In previous morphological studies the distribution pattern of apoB has been determined (Hoff et al. 1977, 1978; Hoff and Bond 1983; Yomantas et al. 1984; Yamauchi and Hoff 1984; Carter et al. 1987). The results of these investigators are confirmed with regard to tissue distribution. The presence of apoB indicates that VLDL or LDL must be present, and from the findings of this study the presence of LDL cannot be excluded. The major new conclusion is that immunoreactivity for apoB

has to be related to the presence of Lp(a) at least in part, since apo(a) and apoB are detected in congruency. One should therefore keep in mind the possibility that Lp(a) is present when apoB is detected in atheromatous lesions.

Walton (1974) first reported the observation of similar distribution patterns for apo(a) and apoB. However, he found it to be "rather unlikely that the finding of apo (a)-immunoreactivity in patients with clinical evidence of coronary artery disease has any special sinister significance". On the basis of our data we can clearly contradict this statement.

In conclusion, this work describes Lp(a) for the first time as an atherogenic particle that can be detected immunologically by the demonstration of apo (a). It has a preferential deposition in atheromatous lesions and should be considered as a lipoprotein that deserves close attention with regard to its diagnostic and therapeutic implications.

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