CYTOPHOTOMETRIC ASSAY OF APOPROTEIN B IN SECTIONS THROUGH THE HUMAN AORTA

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Experiments have shown that persistent elevation of the human blood low-density lipoprotein (LDL) level is a risk factor for the development of atherosclerosis [10]. Quantitative assay of LDL and the study of the character of their distribution in the wall of large human arteries are accordingly particularly interesting. It was shown previously, by immunomorphological [1, 5, 6] and biochemical [1, 7, 8, 15] methods that the intima of adult human arteries contains a large quantity of apoprotein B (apoB), the basic protein of LDL [13]. It has also been shown that some of the LDL are distributed freely among the cells and are readily eluted from homogenized vascular tissue with physiological saline; another fraction of LDL is firmly bound with the connective-tissue fibers of the vascular wall, and their removal requires treatment of the tissue with detergents or proteolytic enzymes [6, 12, 15]. Many attempts to measure the ratio between free LDL and those bound with the vessel wall, using immunoelectrophoresis [1, 5, 8, 9, 15] and radioimmunoassay [12], have led to contradictory results [12]. The causes of the disagreement may be as follows: before measurement of the apoB was isolated from the vessel wall by means of various substances without regard to the nature of the structural elements of the vessel or the course of the atherosclerotic process. In our view, apoB assay directly on histological sections through a vessel may be free from the defects mentioned above. In the investigation described below the possibility of cytophotometric assay of the apoB content in the wall of the human aorta was studied. For this purpose histological sections stained by the immunoperoxidase method using antibodies to apoB were used.

EXPERIMENTAL METHODS

Autopsy material obtained 2-4 h after death from 16 men dying suddenly at the age of 39-72 years was used. Fibrous plaques were excised from the thoracic part of the aorta together with the adjacent part of the vessel, unaffected by atherosclerosis, and also with unchanged regions of the aorta. Frozen serial sections were cut: two strips each of four or five sections were mounted on slides, and some of them were fixed with acetone for 5 min at −20°C. The unfixed sections were covered with 1 ml buffered physiological saline containing 10 mM phosphate buffer (PBS; pH 7.4) and 2 mg/ml of bovine serum albumin, and the sections were incubated for 20-120 min at 25°C, and after fixation, the area of the section was measured. The quantity of apoB in the solution was measured by an immunoenzyme method (ELISA), according to the recommendations in [2]. All the sections were treated for 10 min with methyl alcohol containing 0.5% hydrogen peroxide, and for 30 min with a 20% solution of normal sheep's serum in PBS in order to block the action of endogenous peroxidase and nonspecific binding of antibodies. The apoB was revealed by the indirect immunoperoxidase method, the sections being incubated successively with monospecific rabbit antibodies to human apoB, obtained as described previously [11], and with sheep's antibodies to rabbit IgG, labeled with horseradish peroxidase by the formula in [3]. Each step of the experiment lasted 30 min at 25°C and the concentration of labeled antibodies was 20 μg/ml. As the control of specificity of the reaction, instead of antibodies to apoB, IgG of an unimmunized rabbit in the same concentration was used. Peroxidase was revealed by treatment of the sections with a substrate containing 0.5% diamino benzidine (DAB) and 0.01% hydrogen peroxide in PBS for 10 min at 25°C. The quantity of peroxidase reaction product (DAB/H_2O_2) was measured on an "Opton" cytospectrophotometer, using an IBAS computer with the "Apamos" program. In each case five sections

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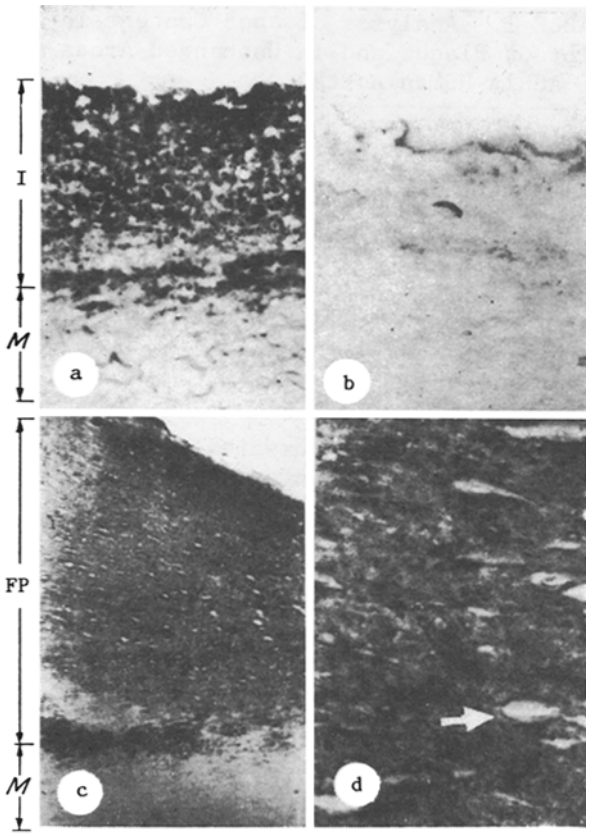

Fig. 1. Distribution of apoB in aortic wall of a man aged 56 years. a) Visually unchanged part of vessel; b) control, sections treated with IgG of an unimmunized rabbit; c, d) fibrous plaque. Arrow indicates cell with a border of connective tissue not containing apoB. Indirect immunoperoxidase method. Magnification: a, b) 40×, c) 16×, d) 100×. M) Media, FP) fibrous plaque.

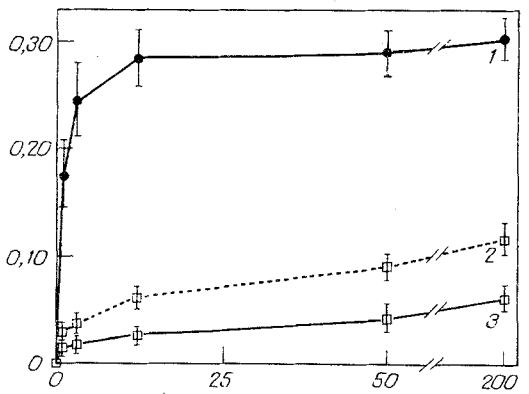

Fig. 2. Dependence of OD on concentration of antibodies to apoB. Abscissa, concentration of antibodies to apoB (in μg/ml); ordinate, OD. 1) Intima, 2) media, 3) control.

TABLE 1. Analysis of apoB Content in Fibrous Plaque and in Unchanged Areas of Adult Human Aorta

Age, years	Affected region	Unchanged areas of vessel	
	fibrous plaque/ media	adjacent intima/media	distant intima/media
56	0,337±0,026	0.431±0.014*	0,433±0,031
	0,064±0,005	0.033±0.003*	0,035±0,008
60	0,385±0,015	0.456±0.022*	0,447±0,029
	0,041±0,007	0.024±0.004	0,033±0,003
65	0,342±0,021	n.d.	0,472±0,027*
	0,052±0,005	n.d.	0,041±0,005

Legend. *p < 0.05 compared with values for fibrous plaque; n.d.) not determined.

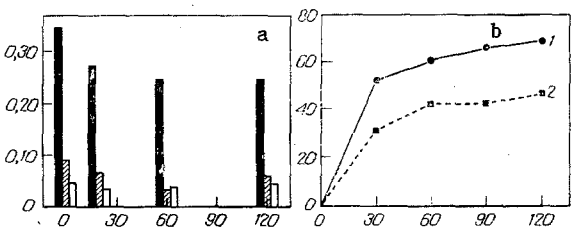

Fig. 3. Changes in intensity of specific staining of sections (a) and elution of apoB into solution (b) from tissue on incubation of unfixed frozen sections 10 μ thick. Abscissa, duration of incubation (in min); ordinate: a) OD, b) quantity of apoB (in μg/cm²). Curves of extraction of apoB from aortic sections of a man aged 65 years (curve 1) and a woman aged 67 years (curve 2) are given in Fig. 3b for comparison. Black columns) intima; obliquely shaded) media; unshaded)• control.

were measured under a magnification of 200. The results were subjected to statistical analysis.

EXPERIMENTAL RESULTS

Preliminary cytospectrophotometric measurements on sections stained by the immunoperoxidase method showed that the optical density (OD) of the reaction product reached a maximum at a wavelength of 470 nm, in agreement with data obtained by other workers [4].

Visual analysis of sections stained by the immunoperoxidase method showed that in all cases, irrespective of age, apoB was distributed diffusely throughout the intima of unaffected regions of the vessel, and only a very small quantity was present in the media of the aorta (Fig. 1a). Sections treated as the control with IgG of an unimmunized rabbit did not give specific staining (Fig. 1b). Compared with the unchanged intima, in the fibrous aortic plaque the intensity of specific staining was somewhat weaker, especially in the roof of the plaque and around those of its cells that were surrounded by connective-tissue membranes (Fig. 1c, d), the ultrastructure of which was described previously [14]. A typical curve showing dependence of the value of OD on the concentration of antibodies to apoB is shown in Fig. 2. Similar curves, reaching a plateau at an antibody concentration of 1.5-3 μg/ml were obtained by analysis of sections of yet another three aortas; for that reason, during the subsequent staining antibodies to apoB were used in a concentration of 3 μg/ml. It must be pointed out that the value of OD of the unchanged intima was 8-22 times higher than that of the media

of the vessel which, in turn, was 1.4-2.6 times higher than the nonspecific background reading in the control.

Comparison of the fibrous plaque with adjacent unchanged areas of the vessel in the same section, stained with the immunoperoxidase method, showed that OD was always higher in an adjacent unchanged area of intima (Table 1). This difference was not due to any particular properties of the intima adjoining the plaque, for it did not differ in OD from the intima of other areas of the unaffected part of the aorta. Variations in the character of distribution of apoB were observed in the tissue of the fibrous plaque: in two plaques a central zone stained almost as intensely as the unchanged intima, and the roof of the plaque, in which the apoB concentration was 2-3 times lower, could be distinguished; in yet another plaque, the distribution of apoB was homogeneous throughout its tissue. In the underlying media of one of the plaques, the apoB concentration was significantly increased.

On incubation of the unfixed frozen sections in PBS, the OD of specific staining of apoB in them was appreciably reduced (Fig. 3a). There was a parallel increase in the quantity of apoB in solution, and this was particularly marked during the first 60 min of incubation (Fig. 3b). In the course of this time, evidently nearly all the free particles of LDL were released from the tissue of the section. Depending on the degree of reduction of OD of the stained sections, the fractions of free LDL and those bound with the vascular well could be determined. This experiment was carried out on three aortas: the fraction of free LDL in them varied from 28 to 42% of the total content of apoB in the vessel wall. A similar value for the fraction of free LDL (34-43%) also was obtained during incubation of unfixed sections of the fibrous plaque in PBS.

The results are thus evidence that the distribution of apoB in sections of a blood vessel can be assayed quantitatively. The validity of earlier communications [1, 8, 14, 15], in which LDL were shown to be located predominantly in the intima of arteries, was confirmed. It can be taken as proven that the media of the human aorta contains a certain quantity of apoB, although this was denied in [5, 6]. Earlier reports [6, 8] stated that the quantity of free LDL in the vascular wall is significantly higher in the unchanged intima than in the atherosclerotic plaque (95 and 42% of the total quantity of apoB respectively). Like other workers [12], we also did not observe this. A relative decrease in the apoB content in the fibrous plaque compared with that in the unchanged intima of human arteries has been reported previously [1, 6, 8]. This phenomenon is linked with active proliferation of connective tissue, and under these circumstances negative correlation was found between the collagen concentration and the LDL content in the tissue [8]. According to our own data, the apoB concentration is particularly low in the roof of the plaque and in the membrane of some of its cells.

It can be concluded that the present investigation illustrates the possibility of quantitative assay of the distribution of antigens in a histological section.

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