ELISA STUDY OF INTERACTION OF LOW-DENSITY LIPOPROTEINS WITH IMMOBILIZED FIBRINOGEN AND FIBRONECTIN

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Accumulation of free and esterified cholesterol in blood vessel walls is a characteristic feature of atherosclerosis. It has been suggested that extracellular deposition of cholesterol esters takes place directly from plasma low-density lipoprotein (LDL) [10]. Immobilization of LDL by their interaction with intercellular connective-tissue macromolecules may be one of the initial stages of the process of lipid accumulation in atherosclerotic involvement [5]. Some workers have demonstrated a link between atherosclerosis and hemostasis. It has been shown, in particular, that mural thrombi and fibrin incrustations may facilitate LDL deposition in vessel walls [8, 11, 15]. Fibronectin is known to participate in reactions of hemostasis and regeneration. Fibronectin filaments are distributed along the course of the fibrin fibers in a fibrin–platelet clot, and are connected to them both by noncovalent adsorption and by covalent bonding, catalyzed by factor XIIIa [3, 7]. During atherogenesis an increase in the concentrations of fibrin and fibrinogen, proportional to the increase in LDL concentration, is found in the intima of the vessels. It has accordingly been postulated that fibrin deposits play a role in LDL binding [9, 12]. The writers showed previously that LDL interacts with fibronectin [2].

This paper describes a comparative investigation of interaction of LDL with immobilized fibronectin and fibrinogen, and also with the fibrinogen–fibronectin complex.

EXPERIMENTAL METHOD

LDL (density 1.019-1.063 g/cm^3) were isolated from serum from donated blood by preparative ultracentrifugation [4] and kept in medium containing 0.05 M NaCl, 0.5 M KBr, and 0.5% EDTA at 4°C for not more than two weeks. Fibronectin was obtained from human blood serum by affinity chromatography on collagen–sepharose followed by fractionation with ammonium sulfate [1]. Fibrinogen, freed from fibronectin, was obtained by chromatography of a commercial preparation of fibrinogen, produced by the Kaunas Bacterial Preparations Factory, on collagen–sepharose. The collagen–sepharose was obtained by immobilizing denatured collagen on CNBr-sepharose 4B. The homogeneity of the resulting preparations was determined by electrophoresis in the presence of sodium dodecylsulfate [6]. The protein concentration was determined from the absorbance at 280 nm. Antisera to LDL, fibrinogen, and fibronectin were obtained by immunization of rabbits [13]. To study interaction of LDL with immobilized fibrinogen and fibronectin, the ELISA technique was used: 1 μg protein (or fibrinogen or fibronectin) in 100 μl phosphate–salt buffer – PSB (0.7 mM K_2HPO_4, 0.17 M NaCl) pH 7.5 was transferred into polystyrene wells and left for 24 h at 4°C. The protein solution was then withdrawn and the wells washed three times with 150 μl PSB (12 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl, 3 mM KCl) pH 7.8 with 1% serum albumin, to abolish nonspecific binding. Into wells covered with fibrinogen or fibronectin, 100 μl of a solution of LDL (from 1 to 10 μg LDL protein in PSB) was poured and incubated at room temperature for 1 h. After incubation the solution was withdrawn, the wells were thoroughly washed with PSB, and 100 μl of antiserum against LDL was added (in a dilution of 1:1600), after which the sample was incubated at room temperature for 45 min. The antiserum was then removed, the wells washed with PSB, and antilipoprotein antibodies bound with adsorbed LDL were determined with the aid of goat antibodies to IgG conjugated with peroxidase ("Cappel," USA). The substrate was 5-aminosalicylic acid (Sigma, USA).

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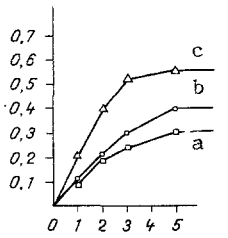

Fig. 1

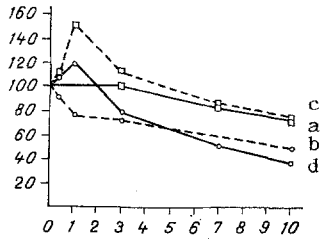

Fig. 2

Fig. 1. Binding of LDL with immobilized fibrinogen (a), fibronectin (b), and fibrinogen–fibronectin complex (c). Abscissa, quantity of LDL in sample (in μg protein); ordinate, optical density at 450 nm.

Fig. 2. Binding of LDL with immobilized fibrinogen (continuous line) or fibronectin (broken line) in the presence of dissolved fibrinogen (a, c) or fibronectin, (b, d). Abscissa, quantity of dissolved fibrinogen or fibronectin (in μg) in samples with LDL; ordinate, binding of LDL (in percent). Binding of LDL from solution not containing fibrinogen or fibronectin taken as 100%.

Development of the color was stopped with 1 M NaOH, and absorbance in each well was measured at 450 nm in an automatic microphotometer (Dynateck, USA). To obtain a complex of fibrinogen with fibronectin, 100 μl of a solution of fibronectin (3 μg in PSB) was added to fibrinogen, immobilized on polystyrene, and the sample incubated for 1 h at room temperature. The quantitative proportion of the proteins in the complex were determined as follows. The antigen (fibrinogen, fibronectin, or LDL), in a dose of 1 μg was introduced into polystyrene wells and allowed to stand for 24 h at 4°C. The difference between the quantity of protein introduced into the wells and the quantity of unadsorbed antigen was used to calculate the quantity of LDL, fibrinogen, and fibronectin bound with the polystyrene. The last value was determined by competitive enzyme immunoassay [14]. Of 1 μg of antigen introduced into the well about 0.4 μg was bound. The optical density at 450 nm (OD_{450}) of solution obtained during enzyme immunoassay of this quantity of adsorbed LDL, fibrinogen, and fibronectin was taken as unity. In the subsequent calculations of the quantity of LDL, fibrinogen, and fibronectin in the complexes, it was assumed that the quantity of bound antigen (between 0 and 0.4 μg) is a linear function of OD_{450} during enzyme immunoassay. To determine binding of LDL, no fewer than 12 assays were done. Binding of LDL with immobilized serum albumin, casein, or gelatin served as an indicator of nonspecific interaction.

EXPERIMENTAL RESULTS

It will be clear from Fig. 1 that LDL bind with fibrinogen and fibronectin immobilized on polystyrene. This binding is concentration-dependent and increases with an increase in the LDL concentration in the incubation medium from 1 to 5 μg. If the fibronectin was adsorbed beforehand on fibrinogen, the binding capacity of these proteins relative to LDL was not reduced. The fibrinogen–fibronectin complex bound more LDL than the two components separately, when added together, calculated from their concentration in the complex: 0.4 μg of immobilized fibrinogen or fibronectin bound with 0.04 μg LDL from a solution containing 1 μg LDL, whereas the complex containing 0.4 μg fibrinogen and 0.2 μg fibronectin bound 0.08 μg of LDL. It can be tentatively suggested that LDL have greater affinity for the fibrinogen–fibronectin complex than for each component separately.

To discover if 3-component interaction between LDL, fibrinogen, and fibronectin could take place, binding of LDL with fibrinogen or fibronectin in the solid phase was studied in the presence of these same proteins, but in solution. LDL (30 μg protein) in 1 ml of PSB was incubated at room temperature for 30 min in the presence of increasing concentrations of fibrinogen or fibronectin (from 5 to 100 μg in 1 ml of the solution of LDL). Next, 100 μl of the incubation mixture was transferred into polystyrene wells covered with fibrinogen or fibronectin, incubated for 1 h at room temperature, after which binding of LDL was determined as described in "Experimental Method."

During incubation of LDL in the presence of low concentrations of fibronectin or fibrinogen (up to 10 μg/ml) binding of LDL with these immobilized proteins increased (Fig. 2). With an increase in the fibrinogen or fibronectin concentrations in the solutions of LDL, binding of LDL with the solid phase was inhibited. Dissolved fibronectin inhibited binding of LDL with immobilized fibrinogen by a greater degree than dissolved fibrinogen (Fig. 2a, d), evidence that LDL have higher affinity for fibronectin than for fibrinogen.

The increase in binding of LDL with immobilized fibrinogen in the presence of dissolved fibronectin and with immobilized fibronectin in the presence of dissolved fibrinogen indicated that a 3-component complex could be formed on the surface.

Serum albumin, used as a control of nonspecific interaction, in the same concentrations as fibrinogen and fibronectin, did not affect binding of LDL.

LDL thus interact both with fibronectin and with fibrinogen; moreover, LDL has greater affinity for fibronectin than for fibrinogen. The formation of a complex of fibrinogen with fibronectin leads to an increase in binding of LDL.

It can be tentatively suggested that the fibrinogen–fibronectin–LDL complexes formed during reactions of hemostasis promote accumulation and deposition of LDL in vessel walls.

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