Lipoprotein-binding proteins in the human platelet plasma membrane

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The binding of homologous plasma lipoproteins to specific receptor proteins in the plasma membrane of human blood platelets was studied by ligand blotting techniques. HDL_3, HDL_2 and LDL showed saturable binding to three bands of 156, 130 and 115 kDa, respectively. This binding was not markedly affected by the presence or absence of Ca^{2+} nor by covalent modification of lysine and arginine residues of the apoprotein moieties. However, it can be almost completely reversed by the addition of heparin or suramin.

Platelet *Plasma membrane* *Lipoprotein receptor* *Ligand blotting* *Modification*

1. INTRODUCTION

The reactivity of blood platelets at least to some degree depends on the amount and composition of the plasma lipids and lipoproteins which they contact in vivo [1–4]. This finding can be partially explained by alterations in the composition of the platelet plasma membrane in exchange with common lipid pools. Isolated platelets, however, are quite rapidly stimulated upon addition of lipoproteins [4–6], suggesting direct interaction between the latter and partial structures of the thrombocytes. This interaction most likely is mediated via specific receptors on the platelet surface. High-affinity binding of both HDL and LDL to isolated intact platelets has been demonstrated [7,8]. Our findings suggest the existence of a single class of binding sites for both subclasses of lipoproteins, which displays a higher binding affinity towards HDL than for LDL ($K_a = 9 \times 10^7$ and 6×10^7 M^{-1}, respectively). HDL_3 and LDL interfere with binding of each other in a noncompetitive manner. VLDL, which is not bound in a saturable manner, has a similar effect. The characteristics of binding and its possible association with atherogenesis make it desirable to investigate the way by which lipoproteins affect blood platelets. Here, the distinct proteins involved in the binding of lipoproteins to the platelet surface have been identified.

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Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; PRP, platelet-rich plasma; ACD blood, acid-citrate-dextrose blood; PBS, phosphate-buffered saline; NC, nitrocellulose; FITC, fluorescein isothiocyanate; DPH, 1,6-diphenyl-1,3,5-hexatriene; CHD, 1,2-cyclohexanedione; TNBS, 2,4,6-trinitrobenzenesulfonic acid

2. EXPERIMENTAL

2.1. *Materials*

Suramin was kindly provided by Bayer-Austria, Vienna. Biotinylated antibodies were from Amersham (England); all other antisera were from Behringwerke (Marburg) or Immuno (Vienna).

2.2. *Preparation of human platelet membranes*

PRP was prepared from ACD-blood within 2 h of collection. After removal of erythrocytes and repeated washing, platelets were finally suspended

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in 0.01 M Tris-HCl, pH 7.5 (0.15 M NaCl, 1 mM EDTA, 4 mM PMSF), to a concentration of 2–4 × 10^6 platelets/μl. The suspension was sonified for a total of 100 s at 0°C (Branson B-30) and further treated following the centrifugation technique of Barber and Jamieson [9] with minor modifications, including the addition of PMSF in all steps, and omitting the last centrifugation step. The suspension medium for the membrane preparation was 15 mM Tris-HCl, pH 7.5, 0.125 M sucrose, 75 mM NaCl, 1 mM PMSF, 1 mM EDTA. Lactic dehydrogenase and acid phosphatase, respectively, were determined to monitor the progress of purification of the membrane proteins. The corresponding values are in good accordance with those obtained by the lysis technique [9]. The preparations were stored in aliquots at −80°C for up to 2 months and thawed at 0°C immediately before use.

2.3. *Plasma lipoproteins*

Human VLDL, LDL, HDL_2 and HDL_3 were isolated by sequential ultracentrifugation [10] and used within 1 week following filtration through Millipore filters (0.45 μm). Concentrations are expressed as protein content, determined by the method of Lowry et al. [11]. Modifications: Acetylated lipoproteins were prepared by the method of Basu et al. [12]. The degree of acetylation of amino groups ranged from 22 to 30% for LDL, and from 62 to 68% for HDL_3, respectively, as determined by titration of free amino groups with TNBS [13]. Labeling with FITC was performed as in [14]. Noncovalent labeling of the lipid phase of lipoproteins with DPH was accomplished as in [15]. Experiments with CHD-modified lipoproteins, prepared according to [16], were performed in borate buffer, pH 7.5. [^{125}I]Iodinated ligands were either prepared by the iodogen method [17] or with the Bolton-Hunter reagent [18]. Biotinylation of LDL was performed following [19]. Identical results were obtained following modification of the carbohydrate residues by the method of Wade et al. [20]. Limited proteolysis was achieved by incubation with proteinase K or trypsin for 1–6 h.

2.4. *SDS-PAGE*

0.1% SDS-PAGE was performed in 8% polyacrylamide slab gels, containing 18% (w/v) glycerol. Samples were prepared by incubation with 2% SDS for 45 min at room temperature without addition of reducing agent.

2.5. *Immunoblotting*

Membrane proteins were transferred to NC according to Towbin et al. [21]. Following blocking with 3% casein, the paper was incubated with the indicated concentrations of lipoproteins in 1% gelatin-PBS for at least 2 h. Biotinylated lipoproteins were visualized by incubation with streptavidin-biotinylated-peroxidase complex [20]. Bound radioiodinated ligands were detected by autoradiography (Kodak X-Omat S). In all other cases the NC papers were incubated with anti-apolipoprotein antibodies (rabbit or goat). The bound antibodies were visualized by ELISA applying either peroxidase-labeled anti-rabbit(goat) antiserum, or biotinylated anti-rabbit(goat) IgG.

3. RESULTS

The binding of native LDL to platelet plasma membrane proteins is shown in fig.1. The amount of lipoprotein associated with individual protein bands in each case obviously is concentration-dependent. In the vast majority of membrane preparations binding to two or three protein bands

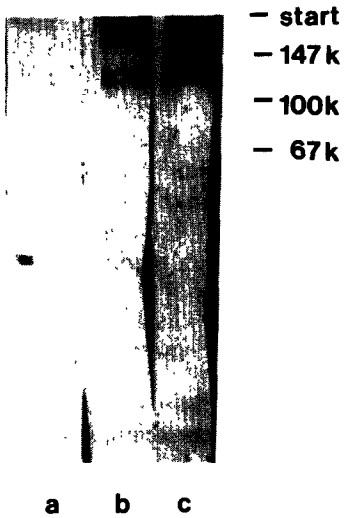

Fig.1. Visualization of binding of human LDL to platelet plasma membrane proteins (140 μg total) transferred to nitrocellulose. Concentration of LDL (μg protein/ml): (a) 0, (b) 4.2, (c) 21.

was observed, the corresponding molecular masses being 156 ± 6, 130 ± 5, and 115 ± 2 kDa. Occasionally, protein bands corresponding to much lower molecular masses also showed abilities to bind LDL. (The relative frequency of the various lipoprotein-binding protein bands is shown in fig.2, reflecting the results with a total of 39 different membrane preparations.) A number of experiments was undertaken to investigate the specificity of the observed binding reactions. Vesicles prepared from phosphatidylcholine with and without cholesterol cannot prevent binding of LDL. The same observation could be made with serum albumin and lipoprotein-depleted plasma. Using biotinylated LDL, however, unmodified LDL clearly competes with binding with the former (fig.3). The specificity of the binding to a limited number of defined protein bands is further confirmed by the virtual agreement of the results obtained with different techniques of visualization of bound ligand, using either biotinylated ligand, or unmodified LDL with two variants of enzyme-linked assay, and, finally, ^{125}I-labeled ligand. Partial proteolysis, however, of LDL greatly reduces its binding affinity.

Following another strategy to test the specificity of interaction, platelet plasma membrane preparations blotted to NC were incubated with LDL, adding increasing amounts of polyvalent anti-apo B antiserum. Complexing of the antigen clearly excluded it from being bound to the membrane proteins (not shown). HDL_2 and HDL_3 were applied as ligands in analogous blotting experiments. Both lipoprotein subclasses apparently bind to the protein pattern capable of binding LDL (fig.4). This finding is further confirmed by the competition experiments illustrated in fig.5. Both members of the high-density class when applied in relative excess can inhibit binding of LDL, and vice versa. This effect, however, is least obvious with the protein band of ~116 kDa. As already revealed by experiments with intact platelets, the reported interactions are independent of any divalent cations, Ca^{2+} in particular. The lack of any significant effect on binding of the presence or absence of Ca^{2+} and the addition of EDTA up to 10 mM is obvious from fig.4. The amount of bound ligand is, however, markedly reduced in the presence of moderate concentrations of heparin and, similarly, by the addition of suramin to the incubation mixtures (fig.3).

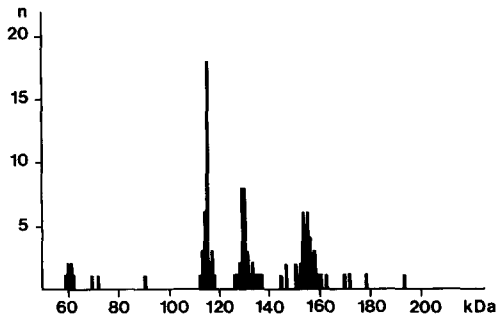

Fig.2. Cumulative frequency of LDL binding to protein bands depending on their relative mobility in SDS-PAGE (indicating the number *n* of positive findings out of a total of 39 experiments).

Fig.3. Influence of various parameters on binding of LDL to transblotted membrane preparations (235 μg protein). Incubation was performed with 2.9 μg/ml LDL alone (a); with the addition of suramin (final concentration 0.07 M, b); 2500 units/ml heparin (h); phosphatidylcholine vesicles (final concentration of phospholipid 225 μg/ml, e); and lipoprotein-depleted plasma, diluted 1:20 with PBS (j), respectively. Lane i shows the visualization of binding of 0.6 μg/ml LDL by means of biotinylated second antibody. 3.5 μg/ml of biotinylated LDL were applied without (c) and with the addition of 52 μg/ml of unmodified LDL (d). CHD-modified LDL (f) and acetylated LDL (g) were applied at protein concentrations of 4.2 and 5.1 μg/ml, respectively.

To investigate the importance of charge and/or the relative importance of lysine and arginine residues of their protein moieties for the specific binding of the various lipoprotein subclasses, experiments with covalently modified ligands were

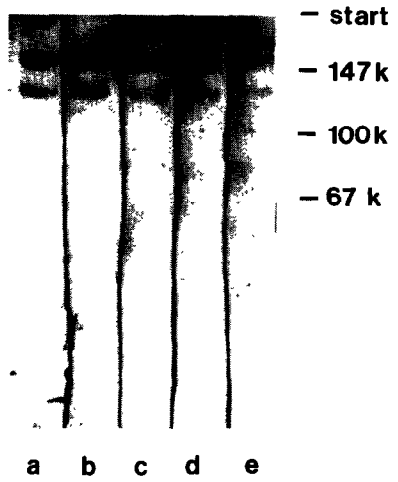

Fig.4. Binding of 3.3 μg/ml LDL with no additions (a), in the presence of 2 mM Ca^{2+} (b), and presence of 10 mM EDTA (c). Binding of 5.1 μg/ml HDL_3 (d) and 2.8 μg/ml HDL_2 (e).

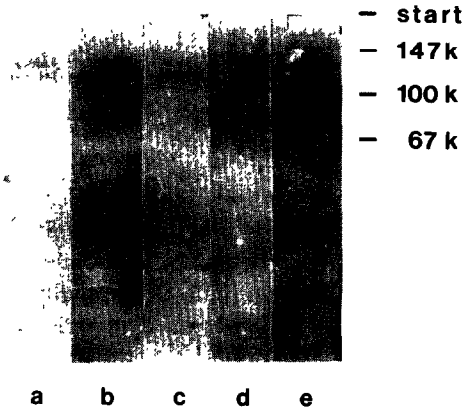

Fig.5. Binding of 3.6 μg/ml LDL (a), in the presence of 16 μg/ml HDL_2 (b), 25 μg/ml HDL_3 (c), 78 μg/ml HDL_2 (d) and 125 μg/ml HDL_3 (e), respectively.

undertaken. Modification with CHD and by acetylation, respectively, has only a minor effect on ligand binding (fig.3f,g). The same applies to the coupling to lysine side chains of rather large chromophoric groups (by introducing dansyl groups or FITC), at least at the degree of modification obtained by us. Similarly, labeling with DPH of the lipid moiety of the various lipoproteins does not interfere with their binding to the protein bands cited above (not shown).

4. DISCUSSION

In agreement with binding data obtained with intact thrombocytes, lipoproteins with buoyant density greater than 1.006 g/ml obviously bind to the same set of individual membrane proteins. The specificity of this observed binding is thus of critical importance. We have shown that only intact lipoproteins on the one hand, and intact plasma membrane proteins on the other, are capable of interacting sufficiently. Binding cannot be achieved by lipid vesicles alone, nor by lipoproteins significantly changed in their apoprotein structure, nor after complexing the latter with polyvalent anti-apoprotein antibodies. Saturability of the binding and inhibition of binding by excessive amounts of competing lipoproteins (including VLDL) deliver additional evidence for the biological significance of the described phenomena.

The existence of at least three different protein bands revealing binding affinity towards plasma lipoproteins either reflects heterogeneity of binding sites or partial degradation of a single (glyco)protein. From binding isotherms nothing in favour of the former possibility can be derived, but this cannot, of course, rule out microheterogeneity of receptor sites. Aviram et al. [22] proposed a model comprising both high- and low-affinity sites for LDL. Their statements are hard to assess, however, since most of their data reflect 'accumulation' of lipoproteins rather than binding due to the rather long times of incubation applied. Additionally, the effects of various lipoproteins (native and modified) described by them in part are contradictory [4,22,23]; the experiment demonstrating replacement of bound ^{125}I-labeled LDL by unlabeled LDL lacks any indication of biphasic binding behaviour [22]. According to our interpretation there still is no direct evidence of the mutual existence of two different classes of receptor sites. The existence of more than one single band showing affinity most likely is caused by partial hydrolysis. The finding that the band of 115 kDa shows some nonspecific binding, and the absence of smaller bands in most preparations, are in support of this interpretation. Very recently, Hassall and Bruckdorfer [24] reported the visualization of a single protein binding ^{125}I-LDL, obtained from a differently prepared platelet mem-

brane fraction. As far as the biochemical properties are concerned, no differences can be detected between the various binding proteins. None needs Ca^{2+} or any other divalent cation to bind any class of lipoprotein. Covalent modification of positively charged amino acid side chains has little or no effect at all. Even the modification with rather bulky chromophoric groups involves little hindrance for binding. The protein(s) under investigation thus obviously do not depend on the binding sequence responsible for binding of apoB and apoE in various tissues [25,26]. Some similarity appears to exist with receptor proteins for HDL in cultured rat and porcine hepatocytes, and in rat intestinal mucosa, respectively [27,28].

Major controversy exists with respect to the biological significance of the observed high-affinity binding of lipoproteins [22,29]. Most of the effects plasma lipoproteins have been reported to exert on platelet activity are observed at concentrations significantly higher than those leading to saturation of high-affinity binding sites. However, this does not take into account the interference displayed by different classes of lipoproteins when simultaneously present, as in vivo. We have shown that competition for sites leads to decreasing apparent affinities, which in turn means that saturation of lipoprotein receptors will not occur at concentrations of ligands markedly below the range of physiological lipoprotein concentrations. Even then, variations in the relative amounts of the different classes of lipoproteins consequently would lead to changes in the pattern of bound ligands. We propose the existence of a single type of receptor protein assuming different functional forms depending on the nature of the bound ligands. This hypothesis is currently being tested with solubilized receptor protein.

Independently, differences with respect to the chemical constitution of the lipoproteins used in different sets of experiments may account for some results exhibiting poor reproducibility. The capacity to bind 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet-activating factor), in particular, as documented by Kulikov and Bergelson [30], could give rise to activation of platelets with complex kinetics. The amount of lipid peroxides within the lipoproteins, on the other hand, largely depending on the method of preparation, shows some negative effect on specific binding (unpublished).

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