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# LOW DENSITY LIPOPROTEIN BINDING TO HUMAN PLATELETS: ROLE OF CHARGE AND OF SPECIFIC AMINO ACIDS

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SUMMARY: Many human and animal cells possess cell surface binding sites, specific for low density lipoproteins. Human platelets are similarly endowed with specific low density lipoprotein receptors. Using chemical modofications of amino acid residues on the low density lipoprotein molecule, we have studied the role of charge and specific amino acids on the binding process. The interaction of the modified low density lipoprotein preparations with gel-filtered platelets and with glass beads was compared. Both cyclohexanedione treated and aceto-acetylated low density lipoprotein did not bind to the platelet surface. However, azo-arsanilated low density lipoprotein bound to the platelets in a manner similar to the binding of native lipoprotein. Cyclohexanedione treated lipoprotein was the only preparation which did not bind to glass beads. The importance of both the presence of the positive charge on the lipoprotein molecule and the availability of specific amino acid residues (arginine and lysine but not tyrosine and histidine) for low density lipoprotein-platelet interaction was thus demonstrated.

Low density lipoprotein (LDL) binds to cells in culture and initiates events which regulate intracellular cholesterol metabolism (1). LDL also exhibits apparent specific binding to glass beads (2), but the latter clearly cannot internalize or metabolize the lipoprotein. Thus the interaction of lipoproteins with cells must be defined in terms which go beyond simple binding. Platelets which are unable to synthesize cholesterol de novo (3) have also been shown to possess specific LDL receptors (4). We have compared specific LDL binding to human platelets with LDL binding to glass beads

Abbreviations: LDL, low density lipoprotein; CHD-LDL, cyclohexanedione treated LDL; GFP, gel-filtered platelets; PRP, platelet-rich plasma.

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and have observed the effects of altering LDL surface charge by blocking specific amino acid residues. We have shown that LDL interaction with platelets is similar to the LDL interaction with fibroblasts (5), but differs from LDL binding to glass beads.

## MATERIALS AND METHODS

RPMI-1640 medium was purchased from Grand Island Biological Company, N.Y. Sodium $^{125}I$ in NaOH was obtained from New England Nuclear, 1,2 cyclohexanedione from Aldrich Chemical Co., and Diketene from Sigma. Glass beads (75-150$\mu$ Type 1-W) were purchased from Sigma and Bio-Gel A-50-m from Bio-Rad laboratories.

### Isolation and characterization of LDL

Human LDL was prepared from plasma by heparin/manganous-chloride precipitation (6) followed by ultracentrifugation in a discontinuous density gradient in a SW41 rotor. The LDL was diluted to 5mg of protein/ml, dialyzed against 0.15M NaCl, 0.01% EDTA, pH 8.6 and characterized by paper electrophoresis, immunodiffusion and immunoelectrophoresis. Gel chromatography on Sepharose 2B column showed no aggregation of the LDL.

### LDL iodination

Human LDL was iodinated by the iodine monochloride method of McFarlane as modified by Bilheimer et al (7,8). The labelled LDL was dialyzed extensively against 0.15M NaCl, 0.01% EDTA pH 8.6 and sterilized by filtration (0.22$\mu$). It too was unaggregated as judged by gel filtration on Sephrose 2B.

### Platelet separation and incubation

Venous blood was collected through siliconized needles into plastic syringes. Nine volumes of blood were added to one volume of 3.8% sodium citrate in plastic tube. Samples were spun at 100 x g for 10 min, at 23°C, and the platelet-rich plasma (PRP) had platelet counts of 250,000 - 400,000/$\mu$1. The PRP (5 ml) was chromatographed on a 27 x 90 mm column of Bio-Gel-A-50-m that had been pre-equilibrated with RPMI-1640 medium. One ml fractions were collected during elution at a flow rate of 1 ml/min, and the gel-filtered-platelets (GFP) recovered in over 60% yield as a visible turbid peak. As determined by microscopic examination and in vitro aggregation studies, this platelet preparation was intact and free of LDL as evidenced by double immunodiffusion in agarose plates. Platelet (5-8 ml, 2000,000/$\mu$l) were incubated in RPMI-1640 medium containing 10% lipoprotein deficient serum (LPDS) at 23°C for up to 3 hours.

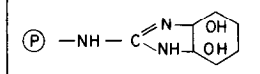
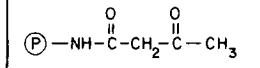
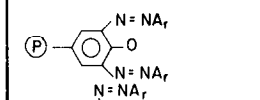
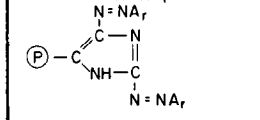
### Assays for LDL accumulation

The accumulation of $^{125}I$-LDL by platelets was studied in two different ways.

1) Using a modification of the method described by Ho et al (9). The platelet aliquots were washed with one ml of modified Ringers solution (107mM NaCl, 20mM $NaHCO_3$ 4mM KCl, 2mM $Na_2SO_4$, 2mM EDTA pH 7.2) and after 4 washes the resuspended pellet (0.4 ml) was overlayered on 0.8 ml of fetal calf serum and spun

Table I

Chemical Modification of LDL

| Modifying reagent | Residue modified | Structural modification |
|---|---|---|
| Cyclohexanedione | Arginine |  |
| Diketene | Lysine |  |
| Diazotized-arsanilic acid | Tyrosine |  |
| | Histidine |  |

Ⓟ — Protein moiety

$A_r$ — Arsanilic acid

down again for 2.5 min at 8,000 x g in a minicentrifuge (Eppendorf 3200). The supernatant was removed and the pellet counted in a scintillation counter (Packard Model 3385). LDL binding to glass beads was measured in a similar way.

2) Aliquots of cell suspension were spun down (as described above) to remove unbound $^{125}$I-LDL and the resuspended pellet (0.4 ml) was rechromatographed on 27 x 90 mm plastic columns of Bio-Gel A-50-m as described above.

Chemical modification of LDL

1) Cyclohexanedione treated LDL (10). One ml of LDL (5 mg/ml) was added to 2 ml of 0.15M 1,2 cyclohexanedione in 0.2M sodium borate pH 8.1 and incubated at 35°C for 2 hours. The modified LDL was dialyzed against EDTA-saline pH 8.6 and filtered through a 0.22$\mu$ millipore filter before use.

2) Aceto-acetylated LDL (5). Five mg of LDL in saline were diluted with 2 volumes of 0.3M sodium borate at pH 8.5. 3$\mu$ Moles of diketene were added to the LDL solution, incubated at 23°C for 5 minutes and dialyzed against 0.2M carbonate buffer pH9.5 for 5 hours at 23°C and then for 16 hours at 4°C. The modified LDL was dialyzed against EDTA-saline pP 8.6 and filtered before use.

3) Azo-arsanilated LDL. The LDL was first dialyzed extensively against 0.1M carbonate buffer pH 9.8. Then 2.44 x $10^{-2}$ mmoles of diazotized arsanilic acid prepared by incubation of 1 mmole of recrystallized arsanilic acid with 1mmole sodium nitrite (11) were added slowly with vortexing to 10 mg of LDL in carbonate buffer at 4°C over a 15 minute period. After 4 hours of incubation at 4°C, the modified LDL preparation was dialyzed

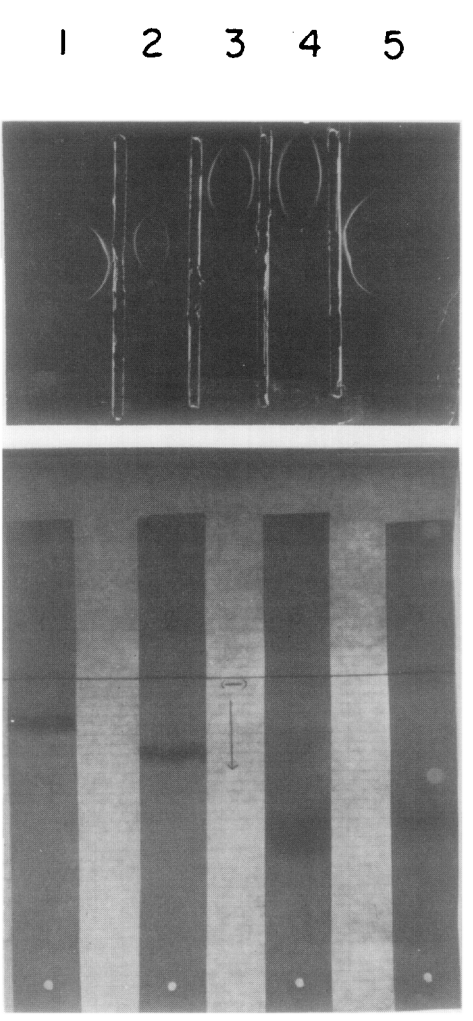



Figure 1. Characterization of modified low density lipoprotein by immuno-electrophoresis and paper electrophoresis.
Upper panel: Immunoelectrophoresis of native and modified LDL against anti-human LDL (in the troughs). The wells contain 1)native-LDL, 2)CHD-LDL, 3)Aceto-acetylated-LDL, 4)Azo-arsanilated-LDL, 5)native-LDL.
Lower panel: Paper electrophoresis of native and modified LDL in albumin containing buffer. The strips contain 1)Native-LDL, 2)CHD-LDL, 3)Aceto-acetylated-LDL, 4)Azo-arsanilated-LDL.

for 7 days against EDTA-saline pH 8.6. The modified LDL was filtered through a 0.22$\mu$ millipore filter before use.

These specific modifications of the amino acid residues of the LDL apoprotein are listed in Table I.

The chemical modification resulted in altering the charge in the lipoprotein, and this was demonstrated in both immunoelectrophoresis and paper electrophoresis. The modified LDL preparations migrated faster than the native LDL (Fig. 1). Both aceto-

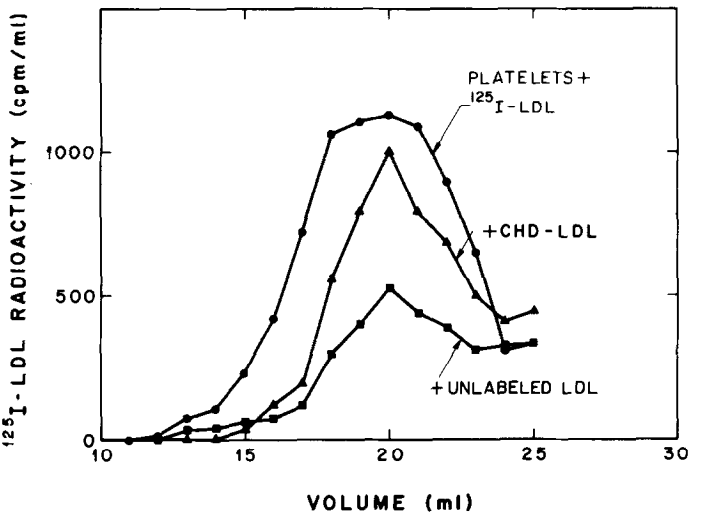



Figure 2. Gel filtration chromatography of human platelets incubated with $^{125}$I-LDL : effects of excess CHD-LDL. GFP (200,000/$\mu$l)were incubated with 50 $\mu$g/ml of $^{125}$I-LDL (340 cpm/ng of protein) for 2 hours at 23°C with no addition (●) or with addition of 500 $\mu$g/ml of native LDL ( ■ ) or 500 $\mu$g/ml of CHD-LDL ( ▲ ). The platelets were spun down and the resuspended pellet rechromatographed on 27 x 90 mm plastic columns contained Bio-gel A-50-m. Fractions were collected and counted in the gamma counter.

acetylated LDL and azo-arsanilated LDL are similarly negatively charged relative to CHD-LDL and native LDL.

## RESULTS

Fig. 2 illustrates the LDL-human platelet interaction using the preparation of gel-filtered platelets (GFP). The additional excess native LDL, greatly reduced the amount of radioactivity associated with the platelets, whereas excess CHD-LDL decreased the amount of platelet bound $^{125}$I-LDL only slightly.

Similar results were obtained on determining the platelet accumulation of LDL after repeated washings of the platelets (Method 1) (Fig. 3).

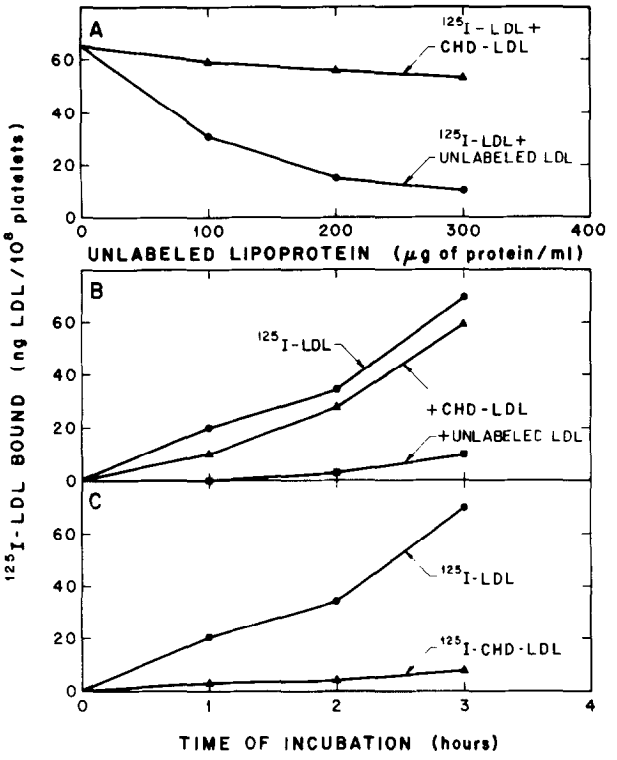



Figure 3. CHD-LDL binding to human platelets.
A. Ability of native human LDL ( ● ) and CHD-LDL ( ▲ ) to compete with $^{125}$I-LDL for accumulation by platelets. Platelets (200 000/μl) were incubated at 23°C for 2 hours with $^{125}$I-LDL (25 μg /ml) and increasing concentration of unlabelled native LDL or CHD-LDL.
B. Time curve for $^{125}$I-LDL (50 μg/ml) accumulation by platelets without ( ● ) or with addition of 500 μg/ml of unlabelled native LDL ( ■ ) or unlabelled CHD-LDL ( ▲ ).
C. Accumulation of $^{125}$I-LDL (50 μg/ml) or $^{125}$I-CHD-LDL (50 μg/ml) by human platelets. The specific radioactivity of the $^{125}$I-LDL was 140 cpm/ng of LDL protein.

CHD-LDL unlike native LDL did not compete with $^{125}$I-LDL for platelet binding (Figs. 3A and 3B). This was shown for both varying concentrations of LDL and CHD-LDL and when fixed amount of unlabelled and modified LDL were incubated with platelets and $^{125}$I-LDL for varying times (Fig. 3B). The above was further confirmed by observing that platelets accumulated little iodinated CHD-LDL in comparison with $^{125}$I-LDL (Fig. 3C).

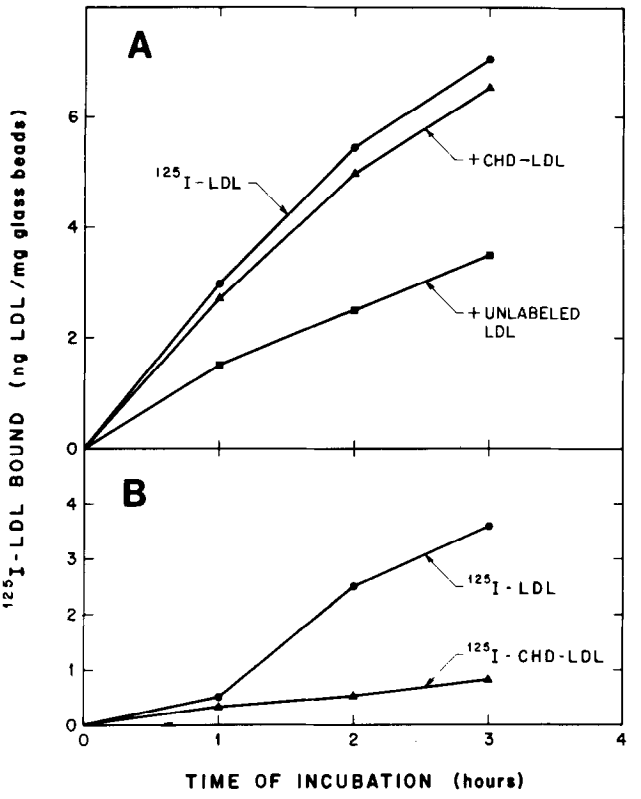



Figure 4. CHD-LDL binding to glass beads.
A. Glass beads (10 mg/ml. 75-150 sigma, Type I-W) were incubated with 50 μg/ml $^{125}$I-LDL (51 cpm/ng) without (●) or with the addition of excess native LDL (500 g/ml, ■) or excess CHD-LDL (500 μg/ml, ▲) for 3 hours with constant shaking at 23°C.
B. 25 μg/ml of $^{125}$I-LDL (●) or $^{125}$I-CHD-LDL (▲) were incubated with glass beads (10 mg/ml) under the same conditions as described for A.

Native LDL, but not CHD-LDL, strongly competed with $^{125}$I-LDL for binding to glass beads (Fig. 4A). Again, on comparing $^{125}$I-CHD-LDL, the binding of the latter to glass beads was minimal (Fig. 4B). Thus native LDL and CHD-LDL behave in a qualitatively similar fashion with regard to binding with platelets and glass beads (Figs. 3 and 4).

Of the modified LDL preparations CHD-LDL had the lowest capability of competing with $^{125}$I-LDL for platelet binding

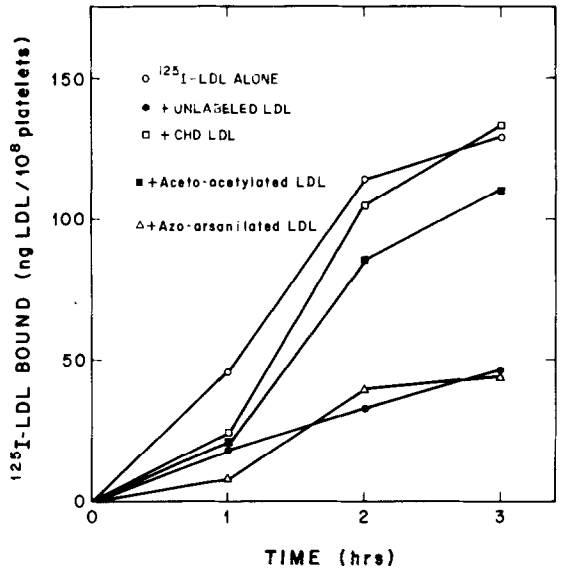



Figure 5. The influence of modified LDL preparations on $^{125}$I-LDL accumulation by platelets. GFP (200,000/μl) were incubated with $^{125}$I-LDL (50 μg/ml, 154 cpm/ng of LDL protein) at 23°C in air with no additions ( ○ ) or with the addition of 500 μg. of protein/ml of native LDL ( ● ), CHD-LDL ( □ ).Aceto-acetylated-LDL ( ■ ) or azo-arsanilated-LDL ( △ ).

(Fig. 5) and aceto-acetylated-LDL competed with the labelled LDL to a limited extent. In contrast, the azo-arsanilated LDL, whose negative charge is almost the same as that of the aceto-acetylated-LDL (but with different modified amino acid residues) was able to compete with the $^{125}$I-LDL to the same extent as unlabelled native LDL (Fig. 5).

In both the control experiment (only 50 μg/ml $^{125}$I-LDL) and with the addition of 500 μg/ml, $10^8$ platelets accumulate 130 ng of $^{125}$I-LDL after 3 hours of incubation. Addition of 500 μg/ml aceto-acetylated-LDL resulted in the accumulation of 110 ng by $10^8$ platelets but only 45 ng of $^{125}$I-LDL are accumulated by the same number of platelets when similar excess native LDL or azo-arsanilated LDL was present (Fig. 5).

The ability of modified LDL preparations to compete with $^{125}$I-LDL for binding to platelets was compared to their ability in competing with binding to glass beads (Table II). 500 $\mu$g/ml of aceto-acetylated-LDL reduced the binding of 50 $\mu$g/ml $^{125}$I-LDL to glass beads by 50 percent but has almost no effect on $^{125}$I-LDL binding to human platelets. CHD-LDL did not bind to either platelets or glass beads, whereas native LDL as well as azo-arsanilated-LDL bind to both and to the same extent.

## DISCUSSION

Using chemical modification of the apo-LDL moiety, we were able to study both the charge effect and the specific amino acid requirements for LDL interaction with human platelets and with glass beads. Our experiments have shown that the platelet LDL interaction is specific for certain amino acid residues and is not simply a charge phenomenon. It is very different from the binding of $^{125}$I-LDL to glass beads (2), connective tissue glycosaminoglycans (12) or dextran sulphate (13) in which the binding process is dependent on the surface charge.

The importance of the positively charged arginine residues on the activity of the LDL binding sites is illustrated by our observation that cyclohexanedione treated LDL did not bind to platelets. This confirmed what has already been reported for human fibroblasts (10). Aceto-acetylation of LDL produced a more negative charged lipoprotein than CHD-LDL, but like the latter, did not affect the $^{125}$I-LDL binding to human platelets. However, the binding of the labelled lipoprotein to glass beads was inhibited by aceto-acetylated LDL but not by CHD-LDL. Azo-arsanilated LDL has a negative charge similar to that of aceto-

Table II. The ability of modified LDL preparations to compete with $^{125}$I-LDL for binding to human platelets and glass beads.

| | $^{125}$I-LDL bound (% of control) | | | |
|---|---|---|---|---|
| | Native LDL | CHD-LDL | Acetoacetylated LDL | Azoarsanilated LDL |
| Platelets | 42.1 | 100 | 98.5 | 41.2 |
| Glass Beads | 38.5 | 98.5 | 49.2 | 37.5 |

Gel-filtered platelets (200,000/μl) or glass beads (10 mg/ml) were incubated with $^{125}$I-LDL (50 μg/ml, 200 cpm/ng protein), for 2 hr at 23°C with no addition or after adding either 500 μg/ml of unlabelled, native or modified LDL preparations.

The control values (no addition of unlabelled LDL's) were 101 ng LDL per mg platelet protein and 6.5 ng LDL per mg glass beads.

acetylated LDL. Nonetheless, it bound to platelets just like native LDL (Table II) (Fig. 5).

We can thus conclude that arginine and lysine but not tyrosine and histidine are of importance in the GFP-LDL interaction. Furthermore, our data suggest that the negative charge on the lipoprotein is not the only important criterion governing the interaction, and also emphasize the role of the specific amino acids at the LDL platelet binding site.

$^{125}$I-LDL binding to glass beads is the same at 4°C and 37°C whereas fibroblasts (2) lymphocytes (9) and platelets (4) have been shown to accumulate more LDL at the higher temperature. These differences might result from the ability of the cells to internalize and degrade LDL whereas glass beads can only bind the LDL to its surface. The binding of the negatively charged modified LDL's as well as native LDL to glass beads suggests that LDL binding to platelets differs from LDL binding to glass beads.

The platelets LDL interaction may be important in the modulation of platelet function. Several indirect lines of evidence suggest that high plasma LDL concentration is associated with altered platelet function (14). The interaction between modified LDL and platelets in relation to their cholesterol content is now under investigation in our laboratory.

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