STUDY OF LOW DENSITY LIPOPROTEIN INTERACTION WITH PLATELETS BY FLOW CYTOFLUORIMETRY

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1. Introduction

Low density lipoproteins (LDL), the main cholesterol carriers in human plasma, are known to interact via the receptor pathway with cultured blood and vessel wall cells. Once bound to the receptor on the cell surface, LDL are internalized and delivered to lysosomes. The LDL degradation in lysosomes results in the release of cholesterol and other lipids, utilized for membrane synthesis [1,2].

LDL affects in vitro platelet aggregation and adhesion [3,4]. Platelets isolated from the plasma of patients with hyper-β-lipoproteinemia have an increased sensitivity to aggregation inducers [5]. LDL exhibit specific binding to platelets [6].

We have studied LDL interaction with platelets by flow cytofluorimetry (FCF) [7], using LDL fluorescence labeled with rhodamine isothiocyanate (RITC–LDL).

2. Materials and methods

LDL (1.019–1.063 g/ml) and high density lipoproteins (HDL) (1.063–1.215 g/ml) were isolated from the plasma of healthy donors by differential ultracentrifugation [8]. ^{125}I-Labeled LDL (^{125}I-LDL) with specific radioactivity of 100–300 cpm/ng LDL protein were prepared by the iodine monochloride method [9], using Na^{125}I (Amersham). LDL were RITC-labeled by the method used for conjugating immunoglobins with fluorescein isothiocyanate [10]. Every LDL molecule bound 20–30 RITC molecules as determined by absorbance at $\lambda = 555$ nm. The concentration of LDL is expressed in terms of their protein content.

Platelets were isolated from the plasma of healthy donors by gel filtration on Sepharose 2B [11] in tyrode solution without Ca^{2+} and Mg^{2+}, containing bovine serum albumin (3.5 mg/ml, Sigma) and apyrase (0.2 mg/ml, Sigma).

Gel filtrated platelets ($0.4–0.8 \times 10^8$) were incubated in 0.2–0.4 ml of tyrode solution with the indicated concentration of ^{125}I-LDL or RITC-LDL for 1 h at 37°C in the presence or absence of unlabeled LDL or HDL. The incubation of platelets with ^{125}I-LDL and subsequent washing were done in the presence of 10 mg albumin/ml.

Unbound ^{125}I-LDL was washed off by centrifugation [12]. After two washings ($1000 \times g$, 15 min, 20°C) the resulting pellet was suspended in 0.2 ml tyrode solution and overlayered on 1 ml newborn-calf serum (Gibco) in Eppendorf plastic test-tubes. Then platelets were precipitated, supernatant was discarded, and the pellet was frozen. After freezing the tube bottoms were cut off and the pellet radioactivity was counted in a γ-counter (Tracor Analytic 1197).

Trichloroacetic acid-soluble ^{125}I-LDL degradation products were determined by the method described for lymphocytes [12].

After the incubation of platelets with RITC-LDL immediately before the measurement the suspension was diluted 100–200-fold with tyrode solution. Fluorescence of individual platelets was measured in the flow cytofluorimeter FACS-II (Becton-Dickinson). Fluorescence was excited at $\lambda = 514$ nm and recorded at $\lambda > 580$ nm. From $0.5–1.0 \times 10^5$ platelets, at $3–5 \times 10^3$ cells/s, were analyzed for each histogram. The level of RITC-LDL binding was estimated by the position of the histogram maximum (mode), after subtracting the mode value of the platelets' autofluorescence.

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3. Results and discussion

RITC-LDL–platelet interaction was studied by FCF. After incubation of platelets with RITC-LDL their fluorescence increased as compared with platelet autofluorescence, that evidences RITC-LDL binding to platelets (fig.1).

RITC-LDL–platelet concentration binding curves in the presence and absence of excess unlabeled LDL are similar to those for ^{125}I-LDL (fig.2). RITC-LDL and ^{125}I-LDL binding to platelets is saturable and inhibited by the excess of unlabeled LDL.

As seen in fig.3a even a 2-fold excess of unlabeled LDL inhibits RITC-LDL binding to platelets. Unlabeled HDL at the same concentration little affects RITC-LDL binding. At 20-fold excess of unlabeled HDL inhibits the binding of RITC-LDL, but to a lesser degree than LDL (fig.3b).

Saturation curve for RITC-LDL binding to platelets, inhibition of binding by unlabeled LDL, and a lesser inhibiting effect of unlabeled HDL provide evidence that platelets possess a limited number of binding sites specific for RITC-LDL. Similarity between fluorescence and radioactive labeled LDL concentration binding curves indicates that RITC-LDL and ^{125}I-LDL behave in a similar fashion with regard to binding with platelets.

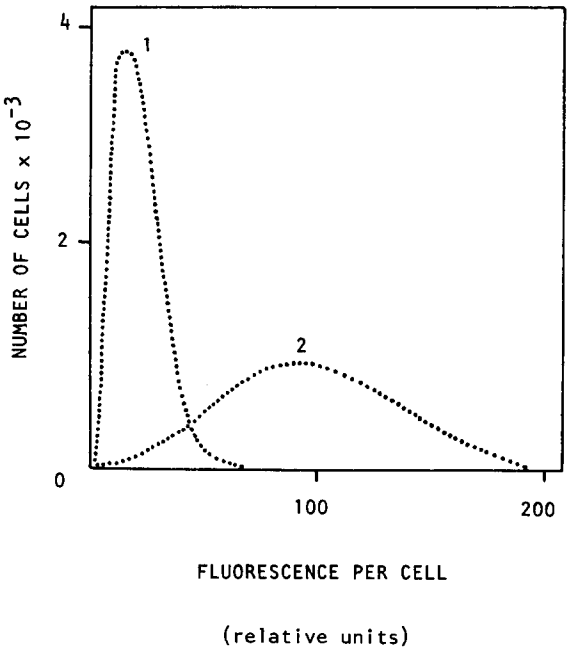

Fig.1. Fluorescence histograms of platelets incubated in the absence of RITC-LDL (autofluorescence) (1) and in the presence of 100 μg/ml RITC-LDL (2).

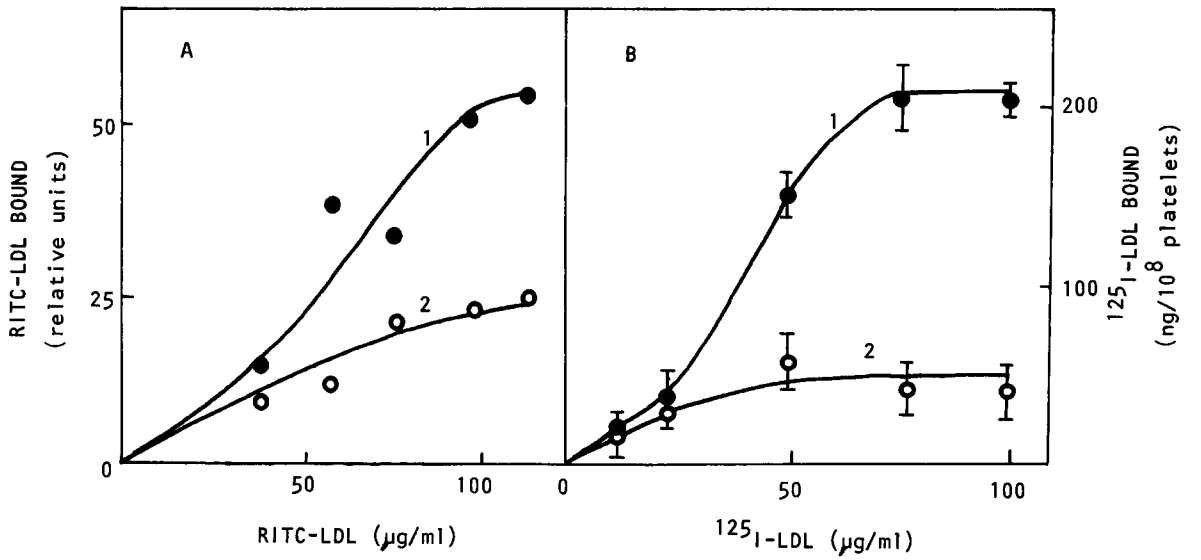

Fig.2. Concentration dependence of RITC-LDL and ^{125}I-LDL binding to platelets. Platelets were incubated with indicated concentrations of RITC-LDL (A) and ^{125}I-LDL (B) in the absence (1) or presence of 20-fold excess unlabeled LDL (2). Mode values of platelet fluorescence histograms are given for RITC-LDL, and means ± standard errors (n = 4) for ^{125}I-LDL.

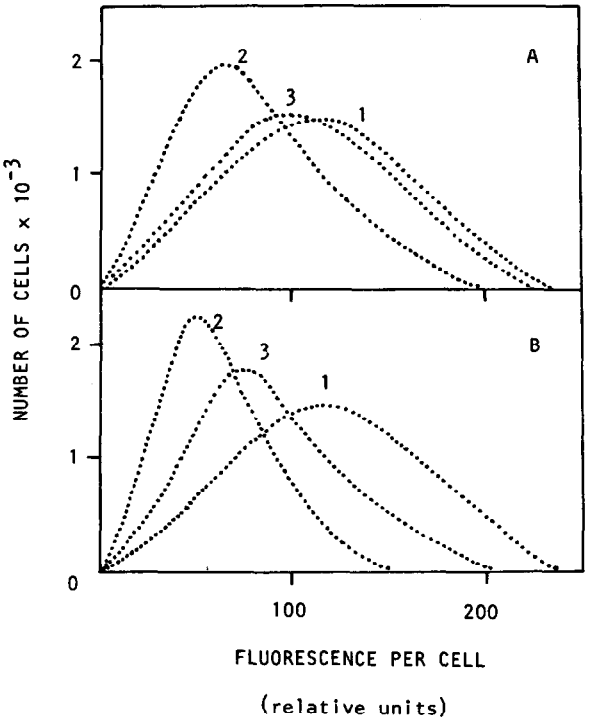

Fig.3. Inhibition of RITC-LDL binding to platelets by unlabeled LDL and HDL. Platelets were incubated with: (A) 100 μg RITC-LDL/ml (1), 100 μg RITC-LDL/ml + 200 μg LDL/ml (2), 100 μg RITC-LDL/ml + 200 μg HDL/ml (3); (B) 100 μg RITC-LDL/ml (1), 100 μg RITC-LDL/ml + 2 mg LDL/ml (2), 100 μg RITC-LDL/ml + 2 mg HDL/ml (3).

The FCF has some advantages in comparison with the radioactive method. It enables:

(1) Evaluation of RITC-LDL binding to an individual cell;
(2) Control of aggregate formation in the platelet suspension under study by simultaneous measurement of fluorescence and light-scattering;
(3) Measurement of only the fluorescence of RITC-LDL associated with platelets without preliminary washing of unbound label.

Reduction of the total amount of cell-bound LDL at 4°C is caused by inhibition of LDL receptor-mediated endocytosis. LDL binding to surface receptors at 4°C is as effective as at 37°C [13]. We have compared the kinetics of RITC-LDL–platelet interaction at 37°C, 20°C and 4°C (fig.4). At all temperatures kinetic binding curves reach their plateau during 5–15 min. Temperature decrease from 37–20°C does not affect total RITC-LDL binding level, though temperature decrease to 4°C reduces it 4-fold. When a platelet suspension is heated, after incubation at 4°C, the platelets exhibit the same ability to bind RITC-LDL as at 20°C and 37°C. By analogy to other cells we suggest that the decrease of total RITC-LDL binding level at 4°C is caused by inhibition of LDL incorporation into platelets.

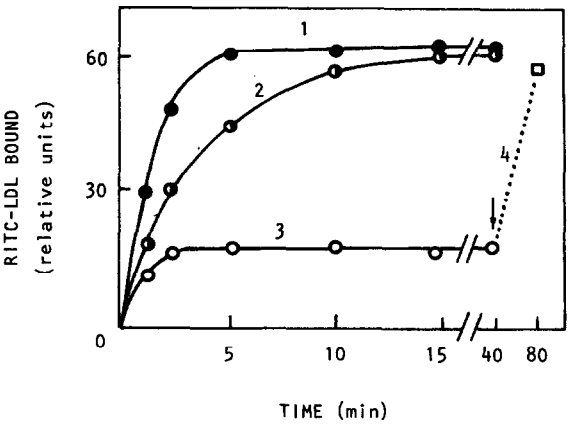

Fig.4. Temperature effect on the kinetics of RITC-LDL binding to platelets. Platelets were incubated with 100 μg RITC-LDL/ml at 37° (1), 20° (2) and 4°C (3). After the incubation at 4°C the platelets suspension was heated and incubated at 37°C (4). (The arrow indicates the moment of temperature change.) Mode values of platelet fluorescence histograms are given.

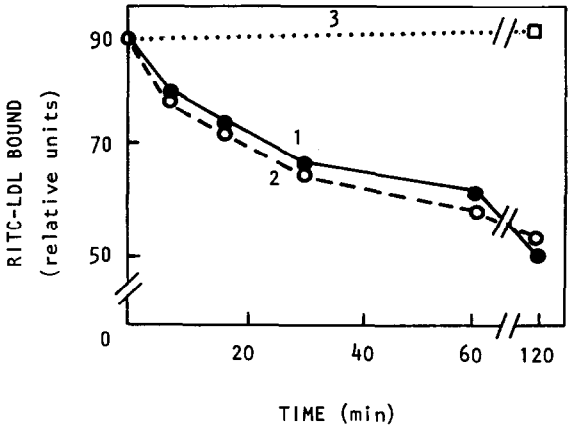

Fig.5. Dissociation kinetics of platelet bound RITC-LDL in the absence and presence of heparin. Platelets were incubated with 100 μg RITC-LDL/ml at 37°C for 1 h, and then at 20°C: (1) after the dilution of initial suspension 200-fold by tyrode solution; (2) after the dilution of initial suspension 200-fold by tyrode solution with 10 mg heparin/ml; (3) without dilution. Mode values of platelet fluorescence histograms are given.

Binding of LDL and its internalization lead to its proteolytic degradation in lysosomes [1,2]. We have not found ^{125}I-LDL degradation products after incubation of 100 μg/ml ^{125}I-LDL with 2×10^8 platelets during 6 h. The content of trichloroacetic acid-soluble radioactive material in the incubation medium in samples with platelets and without them was approximately the same: 5568 ± 562 and 6240 ± 426 cpm, respectively (mean ± standard error, $n = 4$).

Further studies have shown that LDL binding to platelets is partially reversible. The decrease of RITC-LDL concentration after dilution of the incubation mixture leads to a decrease of the amount of platelet-bound RITC-LDL (fig.5). During the same incubation period the RITC-LDL binding level in the initial undiluted suspension does not change. Since platelets do not degrade LDL the decrease of binding level can be due only to the release of bound RITC-LDL into the medium (dissociation). Heparin is known to release LDL bound to the receptors on the cell surface but not to affect the internalized LDL content [13]. Heparin does not affect the dissociation of RITC-LDL bound to platelets (fig.5). Both the absence of the heparin effect and the decrease of binding level at 4°C permit one to assume that bound LDL are incorporated into the platelets.

4. Conclusions

FCF has been used for investigation of LDL–platelet interaction. We have shown that fluorescently labeled RITC-LDL specifically bind to platelets in the same manner as ^{125}I-LDL. A decrease of RITC-LDL binding level at 4°C in comparison with the level observed at 20°C and 37°C, and the absence of heparin effect on the dissociation of bound RITC-LDL indicate that LDL are incorporated into the platelets. Platelets unlike other cells do not degrade LDL. The reversible character of LDL binding suggests that platelets can facilitate in vivo the regulation of LDL distribution in the blood.

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