Reconstituted High Density Lipoprotein (rHDL) Modulates Platelet Activity In Vitro and Ex Vivo

Peter G. Lerch, Martin O. Spycher, Jan E. Doran

From the ZLB Central Laboratory, Blood Transfusion Service SRC, Bern, Switzerland

Summary

A reconstituted high density lipoprotein (rHDL) prepared for clinical use was tested for its influence on platelet activity modulated by various stimuli. In a first series of in vitro experiments, rHDL was added to blood in a concentration series, and platelet rich plasma (PRP) was isolated. Platelets were stimulated with arachidonic acid, collagen, epinephrine or ADP, and platelet aggregation was assessed. rHDL mediated a dose dependent inhibition of the platelet activity. With purified platelets rHDL inhibited the release reaction induced by collagen, but not by thrombin, as measured by CD62P (P-Selectin) expression on the plasma membrane. Ex vivo experiments were performed with PRP from volunteers, previously infused with 25 mg rHDL/kg body weight and 40 mg rHDL/kg body weight, respectively. Platelet activity in PRP was assessed before, and up to 30 h after the end of the HDL infusion. A transient inhibition of the platelet aggregation induced by arachidonic acid and collagen was observed which was more pronounced in the group receiving 40 mg rHDL/kg body weight. In both groups of experiments, in vitro and ex vivo, the inhibition of the platelet activity was also dependent on the stimulus used.

Introduction

A growing body of evidence demonstrates the influence of plasma lipoproteins on platelet function: epidemiological studies have described LDL as a risk factor for arterial occlusive disorders (1), whereas a negative correlation was found with respect to HDL plasma concentration (2). Since platelets are involved in the progress of atherosclerosis, and direct as well as indirect actions of lipoproteins on platelets occur (3), these interactions may be important factors controlling the progress or outcome of the disease.

Lipoproteins also play a role in septic shock: significant changes of plasma lipoprotein concentrations are observed in septicemia. Lipoprotein levels in plasma influence the course of the disease. Both elevated endogenous lipoprotein levels (4) or exogenous lipoproteins, e.g. in the form of intravenously applied rHDL (5), modulate physiological parameters in septic shock models.

Recently, rHDL has become available which can be produced in a quality suitable for clinical applications (6). Among the prospective indications for such a product are the prophylaxis or therapy of septicemia or disorders related to atherosclerosis. In both cases, hyperreactive platelets may contribute to the exacerbation of disease processes. Several observations raise the possibility that HDL′s or related compounds modulate platelet function and attenuate the susceptibility of platelets to different agonists. Consequently, rHDL was tested for its effects on platelet activity, first in vitro and subsequently ex vivo, in order to gain more insight into mechanisms of lipoprotein-platelet interactions, and how these interactions could be made available for therapy and/or prophylaxis.

Platelet aggregation in PRP and the release reaction of purified platelets were measured as parameters of platelet activity. PRP or purified platelets were either incubated in vitro with rHDL, or PRP was isolated from volunteers, previously infused with rHDL. In both sets of experiments, indications of a dose dependent inhibition of the platelet reactivity to certain stimuli were observed.

Materials and Methods

Collagen (Collagenreagent Horm) was purchased from Nycomed Arzneimittel (München, Germany). Arachidonic acid was obtained from Bio/Data Corporation (Horsham, PA, USA). Before use it was diluted with 0.9% NaCl to the appropriate concentration. Stimuli were kept on ice during the experiments. Other reagents were of analytical grade. Production and properties of clinical grade rHDL was described earlier (6, 7). In short, apolipoprotein A-I (apoA-I) was isolated from human plasma fractions to a purity of approximately 90% and pasteurized for 10 h at 60°C. Reassembly of lipoprotein particles was performed by cholate dialysis using soy bean phosphatidylcholine (PC) and sodium cholate, and a molar ratio of apoA-I:PC of approximately 1:150 was obtained. The product was lyophilized in the presence of sucrose and fulfilled all criteria for a parenteral drug. ApoA-I was analyzed by nephelometry on a Behring Nephelometer Analyzer (Marburg, Germany). Phospholipids and HDL-cholesterol were determined using enzymatic color reaction kits (Boehringer Mannheim, Germany) on a Hitachi 704 Clin. Chem. Analyzer.

Platelet aggregations were measured in an Aggregation Profiler PAP-4, Bio/Data Corporation (Horsham, PA, USA). For ex vivo experiments, platelet concentration was determined by a Roche 5DIFF/HELIOS analyzer combination, and for in vitro experiments in a Sysmex Microcellcounter F-800, Toa Medical Electronics Co. (Kobe, Japan). Blood samples were collected into sodium citrate tubes (10 ml Monovette 9NC, containing 1 ml 0.106 mol/l Na-citrate; Sarstedt, Nümbrecht, Germany). Platelet rich plasma (PRP) was obtained by centrifugation for 10 min at 300 × g at room temperature. Platelet poor plasma was obtained by centrifugation of the remaining blood fraction for 10 min at 1500 × g. The platelet concentration was adjusted for aggregation experiments to 250,000 ± 25,000 platelets/µl. Aggregation was performed in siliconized glass tubes with a total volume of 210 µl PRP including 10 µl stimulus. The temperature was set at 37°C and PRP was stirred with a magnetic stirrer at 1000 rpm. Previous to the addition of the stimulus, the platelets were incubated for 2 min under these conditions. Platelet aggregation was recorded until the maximum aggregation was obtained, usually within 5 min. Platelet aggregation experiments were performed within 2 h after blood sampling. The results are expressed as maximal aggregation obtained (%) or as slope values as computed by the Aggregation Profiler. For in vitro platelet aggregations, platelet activity was assessed after a 30 min incubation of citrated blood at 37°C with rHDL or buffer (10% sucrose). Thereafter PRP was isolated and aggregation was performed as described above. Threshold stimulus concentrations were determined in controls without HDL.
In vitro platelet release reaction with purified platelets was determined in an experiment by analyzing the increase of surface expression of CD62P. PRP was obtained after centrifugation of citrated blood for 15 min at 200 × g at room temperature. After adjusting the pH to 6.5-6.8, PRP was incubated at 37°C for 30 min. Platelets were washed once with wash buffer (17 vol Hanks’ Balanced Salt Solution (HBSS) containing 3 mg/ml bovine serum albumin (BSA) + 2 vol 250 mmol/l citrate + 1 vol 200 mmol/l N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) pH 6.8) by centrifugation at 2000 × g for 15 min and resuspension. Platelets were washed a second time with wash buffer lacking citrate. Finally platelets were taken up in the latter buffer at a concentration of 300,000 platelets/ml and left at room temperature until use. Before incubation with rHDL, CaCl₂ and MgCl₂ were added to adjust divalent cation concentration to 1 mmol/l each, and 200 mmol/l HEPES buffer pH 7.8 to adjust pH to 7.2-7.4. After addition of rHDL, platelets were incubated at 37°C for 30 min without agitation to avoid aggregation. Then increasing amounts of collagen or thrombin were added and incubation was continued for another 20 min. Platelet concentration was 200,000/ml during this incubation. At the end of the incubation 20 ml of platelet suspension were added to 1 ml cold HBSS containing 5 mmol/l EDTA and 1 mg/ml BSA to stop activation. Platelets were centrifuged at 4°C at 2000 × g for 15 min and then stained by incubation with saturating amounts of fluorescent anti-CD62P antibody (phycoerythrín labeled; Becton Dickinson, Mountain View, CA) for 30 min at 4°C. After washing, platelets were resuspended in sheath fluid and analyzed on a Facs Calibur flowcytometer (Becton Dickinson, Mountain View, CA).

Clinical Study

rHDL was used in an open-label Phase I study in healthy male volunteers. After appropriate signed consent, six volunteers were infused over a 4 h period with 25 mg rHDL/kg body weight (BW), and four volunteers with 40 mg/kg BW (the rHDL dose was based on the protein concentration). The study was performed at Clin-Pharma Research AG (Birsfelden, Switzerland), and approved by an International Ethical Committee. Blood samples were taken for platelet aggregation at 0 h (prior to rHDL administration), 4, 6, 12 and 24 h. Additional samples were tested at 8 and 30 h from 3 volunteers receiving 25 mg rHDL/kg BW, and from all volunteers receiving 40 mg rHDL/kg BW. Platelets were stimulated with collagen and arachidonic acid at a threshold concentration individually defined at time 0 h; the stimulus concentration was then kept constant throughout the experiment.

Statistical Analyses

Aggregation results and apoA-I concentrations are presented as means ± SEM. Statistical differences of platelet aggregations in the clinical studies were determined using Analysis of Variance and Duncan’s multiple range test with significance set at p < 0.05. Pearson correlation coefficients were calculated using all data obtained over time periods from 0 to 30 h post beginning of rHDL infusion. All probabilities are two-tailed.

All statistical analyses were performed using SPSS/PC software.

Results

Effect of rHDL on Platelet Activity In Vitro

Platelet aggregation: In preliminary experiments the effect of rHDL on platelet activity was tested in vitro in three independent experiments. Citrated blood was incubated with 0-2 mg rHDL/ml, PRP was subsequently isolated and used for aggregation experiments. To assess the threshold concentration of the stimulus, the control platelet suspension without rHDL added was used. The same concentration of the agonist was used for subsequent aggregation experiments in the presence of various rHDL concentrations. With these experiments we could show a dose-dependent inhibition of platelet aggregation (Fig.1): ADP induced aggregation was reduced from 87% to 33% if rHDL concentration was increased from 0 to 2 mg/ml (means of maximal aggregations); for arachidonic acid a reduction from 81% to 28%, and for collagen from 78% to 28% was measured. The inhibition of the epinephrine stimulated platelet aggregation was less pronounced with a reduction from 78% to 48%; slope values were not significantly influenced in these assays (data not shown). In a control experiment with collagen stimulated platelets, rHDL was replaced with apoA-I. At concentrations between 0 to 2 mg/ml apoA-I, maximum platelet aggregation varied from 79 to 71% (Fig. 1D).

If threshold concentrations of the stimuli were increased, platelet aggregability was gradually restored and inhibitory effects of rHDL on platelets diminished.

Platelet release reaction: As a measure of the release of components of platelet storage granules, the upregulation of CD62P (P-Selectin) on the platelet plasma membrane during platelet activation was determined. CD62P is a constituent of the membrane of α-granules of platelets which becomes exposed on the plasma membrane during the release reaction by fusion of the membrane of α-granules with the plasma membrane. Washed platelets were preincubated without agitation for 30 min at 37°C with rHDL or, as a control, with a BSA solution containing the same amount of sucrose as present in rHDL. Then increasing amounts of collagen or thrombin were added and after a further 20 min incubation at 37°C, activation was stopped and aliquots of platelets were analyzed by flow cytometry. Inhibition of the collagen induced release reaction by 2 mg/ml rHDL was found to be up to 60% and was more pronounced with higher concentrations of the activator (Fig. 2A). With thrombin as activator no rHDL induced inhibition was observed (Fig. 2B).

Modulation of Ex Vivo Platelet Activity after rHDL Infusion

Threshold concentrations of the agonists used for platelet aggregations were determined using the volunteer’s baseline sample. This concentration was kept constant for that individual volunteer throughout
the study. Stimulus concentrations used for the activation of platelets varied between 0.25-1 µg/ml for collagen, 0.5-1 mmol/l for arachidonic acid, 1.5-3 µmol/l for ADP, and 15-30 µmol/l for epinephrine, depending on the volunteer. The results of the aggregation experiments are shown in Fig. 3A (maximal aggregation) and Fig. 3B (slope). The platelet reactivity to a given stimulus concentration showed considerable variability between volunteers, and the volunteer’s platelets reacted differently to the rHDL infusion, reflected by the substantial errors associated with each point (error bars represent SEM). As expected, a correlation between aggregation values and slopes was observed: Significant differences from baseline (p < 0.05) were observed for aggregation values at the same time points for most series measured (Fig. 3B). Platelet aggregation induced by arachidonic acid was inhibited by 35% and 68% with rHDL at doses of 25 or 40 mg/kg BW, respectively. Maximal inhibition was at 6 h; platelet function recovered quickly, returning to normal at 8-10 h. A prolonged inhibition was seen when collagen was used as a stimulus. Aggregation was inhibited by 39% and 57% for the low and high dose of rHDL, respectively. Platelet reactivity only returned to baseline values after 12 to 24 h. For both agonists, aggregation values and slopes correlated within an expected variability. For arachidonic acid stimulated platelets as well as for collagen stimulated platelets, the inhibition correlated negatively with the plasma concentrations of apoA-I, PC, HDL-cholesterol, as well as with the dose of rHDL infused (Fig. 4, Table 1).

Under the conditions applied in the test system, little or no influence in the platelet activity was seen if ADP or epinephrine were used as
stimuli. Neither aggregation values nor slopes differed at any time point significantly from baseline values, for either 25 or 40 mg rHDL/kg BW (data not shown).

Discussion

With the first set of experiments we demonstrated that rHDL is able to modulate platelet activity in vitro. The susceptibility of platelets to several agonists was significantly decreased in the presence of rHDL. In contrast to lipoprotein particles, apoA-I in an equivalent concentration had only minimal effects on platelet activity, demonstrating the importance of the particle structure and conformation. rHDL infusions in volunteers confirmed the inhibitory action of rHDL on platelets as shown by ex vivo aggregations induced with arachidonic acid and collagen. These experiments, however, indicate different mechanisms of action, since the time courses of the inhibition recorded with arachidonic acid do not exactly match those of the collagen stimulation, and the statistical correlations between lipoprotein parameters and either collagen or arachidonic acid were different (Table 1). Whether all trends observed are significant remains to be elucidated by further experiments. For ethical reasons, only a limited total volume of blood was taken from the enrolled volunteers, therefore the number of ex vivo experiments was restricted.

The actions of lipoproteins on human blood platelets have been studied in the past several years. Binding sites for lipoproteins on platelets have been identified, and modulation of platelet activity as a response to binding was investigated in detail, mainly for LDL, modified LDL, and HDL and its subclasses. A picture emerges in which oxidized LDL alone activates platelets, and native LDL, in combination with platelet agonists, enhances platelet activation. Attempts to modulate platelet activity with HDL have led to conflicting results. The statistical correlations between lipoprotein parameters and either collagen or arachidonic acid were different (Table 1). Whether all trends observed are significant remains to be elucidated by further experiments. For ethical reasons, only a limited total volume of blood was taken from the enrolled volunteers, therefore the number of ex vivo experiments was restricted.

The actions of lipoproteins on human blood platelets have been studied in the past several years. Binding sites for lipoproteins on platelets have been identified, and modulation of platelet activity as a response to binding was investigated in detail, mainly for LDL, modified LDL, and HDL and its subclasses. A picture emerges in which oxidized LDL alone activates platelets, and native LDL, in combination with platelet agonists, enhances platelet activation. Attempts to modulate platelet activity with HDL have led to conflicting results. An explanation for observed discrepancies may be sought in the different functions of the HDL₂ and HDL₃ subclasses: HDL₂ inhibited, whereas HDL₃ stimulated platelet function (8). Experiments performed with lipoproteins reconstituted from dimyristoyl phosphatidylcholine and apoE or apoA-I suggested that apoE (8, 9) as well as apoA-I (10) play important roles in the inhibitory effect of HDL in platelet function. Our findings therefore support the notion, that HDL or HDL-like lipoproteins reduce platelet hyperreactivity or modulate their susceptibility to stimuli. A number of interactions of lipoproteins with platelets have been described which may account for the inhibitory effect of rHDL on platelets. A receptor for HDL has been identified as GPIIb-IIIa (CD41, CD61), the predominant membrane glycoprotein complex on the platelet membrane (11). GPIIb-IIIa plays a key role in platelet aggregation and adhesion. The binding of rHDL to this protein complex might therefore have major effects on platelet function. Furthermore, collagen binds to GPⅡa-Ⅰa (CD49b, CD29), and, via von Willebrand Factor and fibronectin, indirectly also to GPIIb-IIIa (12). Consequently, rHDL has the potential for a dual function in the inhibition of collagen stimulated

platelets, namely a displacement of the stimulus from its target, and an interference with a membrane protein essential for optimal aggregation. This is supported by our observation that rHDL, even at low concentrations, interfered with the activation of platelets by collagen. A specific interaction of rHDL with the collagen receptor(s) is supported by the experiment with washed platelets which showed rHDL mediated inhibition of collagen, but not the thrombin induced platelet release reaction. Whether epinephrine and ADP interaction with the respective receptor is inhibited by rHDL, or whether rHDL induced inhibition of epinephrine and ADP induced platelet aggregation in PRP involves secondary signals by matrix proteins, remains to be determined. Reverse cholesterol transport promoted by rHDL and leading to platelet cholesterol depletion, was excluded as a suppressive effect on platelet activity (13). Several indirect effects of lipoproteins on platelet activity are also known: HDL modulates platelet activating factor (PAF) activity by inhibition of its synthesis in endothelial cells (13) or by modulation of the distribution of PAF and PAF-acetyl hydrolase to lipoproteins (14). Furthermore, HDL or rHDL significantly prolong the half-life of prostacyclin (PGI₂), a potent inhibitor of platelet aggregation (15), and the addition of HDL to endothelial cells enhances the generation of PGI₂ (16). These effects may therefore add to a reduced platelet activity in vivo. Finally, HDL decreases platelet function by increasing platelet nitric oxide synthase which leads to an elevation of nitric oxide and cyclic GMP, ultimately resulting in a reduced platelet aggregation and serotonin release (17).

The association of platelets with early and late stages of atherosclerosis has been repeatedly demonstrated (e.g. 18, 19, 20). The antiatherogenic potential of HDL may therefore be explained not only by an enhanced reverse cholesterol transport, but also by a reduction of the platelet activity and its sequelae. Enhanced platelet reactivity also contributes to reocclusion after percutaneous transluminal coronary angioplasty (21). An apoA-I variant was able to reduce detrimental effects of balloon injury in an animal model (22).

Several studies have demonstrated rHDL’s potential in the prevention and treatment of gram-negative septic shock (4, 5, 23-27). Modulation of platelet activity in inflammatory reactions (28, 29) or septicemia (30, 31) may contribute to the mechanisms involved in the beneficial effects of rHDL seen in sepsis studies.

The results of the present study support the concept that rHDL has inhibitory effects on platelets. Experimental, clinical and epidemiological studies suggest various mechanisms of action which could play a role in this inhibition. Reduced platelet activity may add to the antiatherogenic effect of HDL, as well as to the protective effects seen in gram-negative sepsis. Both conditions are complex and multifactorial disorders, in which lipoproteins or rHDL are capable of interfering at multiple levels with the progression of the disease process. The clinical use of rHDL represents a novel therapeutic approach in sepsis, atherosclerosis, or restenosis.

Acknowledgements

We gratefully acknowledge the support of Clin-Pharma Research staff members and B. Arnet during the clinical studies, the excellent practical skills of M. Illi and T. Iff, and the support of J.-J. Morgenthaler.

References


Received January 10, 1997 Accepted after resubmission April 22, 1998