Binding of $^{111}$In-Labeled HDL to Platelets From Normolipemic Volunteers and Patients With Heterozygous Familial Hypercholesterolemia

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High density lipoproteins (HDLs; $d=1.063-1.21$ g/ml) were isolated by ultracentrifugation and radiolabeled with $^{111}$In. The in vitro binding onto platelets from healthy volunteers ($n=15$) and patients ($n=36$) with heterozygous familial hypercholesterolemia (FH) was investigated. Binding was saturable and indicated high-affinity binding sites, which bound $1,882\pm361$ ng protein of $^{111}$In-HDL/10$^9$ platelets (dissociation constant [$K_d$]=$7\pm3$ μg protein/ml) in healthy volunteers and significantly ($p<0.01$) lower amounts in the FH patients ($1,012\pm439$ ng protein of $^{111}$In-HDL/10$^9$ platelets [$K_d=12\pm4$ μg protein/ml]; $p<0.01$). The capacity to displace one half of the bound ligand ($IC_{50}$) amounted to $14\pm3$ μg protein/ml in healthy volunteers and $22\pm9$ μg protein/ml in FH patients ($p<0.001$). Treatment with lipid-lowering drugs (gemfibrozil, alone or in combination with cholestyramine) in 10 patients resulted in an increased HDL binding capacity: before treatment, $1,280\pm883$; after 2 months of treatment, $2,052\pm873$ ($p<0.05$); and after 6 months of treatment, $2,127\pm812$ ng protein/10$^9$ platelets ($p<0.01$). There was a significant ($p<0.001$) correlation between $^{111}$In-HDL binding data and plasmatic lipid and lipoprotein values. Furthermore, those FH patients with the additional risk factors of smoking ($p<0.05$) and hypertension ($p<0.01$) showed significantly lower $^{111}$In-HDL binding onto platelets. The findings indicate specific $^{111}$In-HDL binding sites for human platelets, which may be decreased in patients with heterozygous FH.

Upregulation of HDL binding sites during lipid-lowering medication therapy supports the hypothesis that high-affinity HDL binding is involved in hyperlipemic disorders and is possibly related to the reactivity of platelets. (Arteriosclerosis and Thrombosis 1992;12:849–861)

KEY WORDS • high density lipoproteins • platelets • interaction • familial hypercholesterolemia

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bundant epidemiological data strongly support an atherogenic role for the apolipoprotein (apo) B-100–containing lipoproteins (low density lipoprotein [LDL], intermediate density lipoprotein, and very low density lipoprotein [VLDL]) and an antiatherogenic role for high density lipoprotein (HDL). 1–3

From the discovery that plasma HDL levels are linked to protection from cardiovascular disease, the concept was derived that this lipoprotein acts as a vehicle to transport cholesterol from peripheral cells to the liver. 4–5

The precise mechanism responsible for uptake, however, has not been known until recently. HDL appears to promote the efflux of cholesterol from peripheral cells, such as arterial smooth muscle cells and macrophages. This may be facilitated by the HDL receptor, which has been found on both of these cell types. Data suggest that the binding process is regulated by the free cholesterol content of the cell and thus is upregulated during times of cholesterol excess, 6–7 i.e., opposite to that of the LDL receptor. HDLs that pick up excess free cholesterol by this process or direct exchange with free cholesterol in plasma membranes have been hypothesized to transport this cholesterol to the liver for excretion by a process termed reverse cholesterol transport. 8

Furthermore, the HDL receptor may be modulated by mitogens, such as platelet-derived growth factor, which has been shown to decrease both HDL binding and cholesterol efflux. 9

Although HDL delivers cholesterol to some cells and removes it from others and specific binding sites for HDL have been demonstrated in all tissues and cell types investigated to date (i.e., macrophages, 10 fibroblasts, 11 arterial smooth muscle cells, 5 endothelial cells, 7,12 hepatocytes, 13 adipose tissue, 14 intestinal mucosa cells, 15 gonads, 16 lymphocytes, 17 and platelets 18–20), their significance is not well understood. Positive identification of a classical receptor that recognizes HDL is lacking, and the heterogeneity of HDL in terms of size and composition further contributes to the skepticism about a functional role for the putative receptor.

Since platelet function was described to be altered in patients with familial hyperlipoproteinemia, 21 many efforts have been made to correlate platelet hyperreactivity with elevated plasma LDL cholesterol levels. Fur-
thermore, it was shown that administration of the lipid-lowering drug clofibrate to hypercholesterolemic patients reversed platelet hyperreactivity.\textsuperscript{22} Reports of the effects of HDL on platelet aggregability, however, have been conflicting, ranging from decreasing,\textsuperscript{18,20} to noneffective,\textsuperscript{23,24} to increasing\textsuperscript{22,25} functional responses. In this study we systematically examined the binding, specificity, and interrelations of $^{111}$In-HDL with washed human platelets derived from normolipemic volunteers and patients with familial hypercholesterolemia (FH) before and during hypolipemic drug therapy. The interrelations of $^{111}$In-HDL with platelets from the same patients will be reported separately (I. Virgolini et al, unpublished observations).

**Methods**

**Lipoprotein Isolation and Characterization**

During the course of the studies, the lipoproteins were isolated from 56 blood donors. None of the volunteers had received any medication for at least 3 weeks before blood donation. Blood (60 ml) was always collected through siliconized needles into heparin-coated vials after an overnight fast. Neither pooled plasma nor pooled lipoproteins were used throughout the study. In all blood donors, routine plasmatic lipid concentrations were determined (cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides). Only blood samples from normolipemic volunteers were used for lipoprotein preparations.

Lipoproteins were prepared from fresh plasma by sequential ultracentrifugation by using potassium bromide for density adjustment as described previously.\textsuperscript{26} After an 18-hour ultracentrifugation (L5-75 ultracentrifuge, 40.3 Ti rotor at 40,000 rpm, 10°C; Beckman Instruments Inc., Palo Alto, Calif.), the VLDL fraction was withdrawn and the density of the infranatant was raised by addition of solid potassium bromide to $d=1.063$ g/ml, where

$$KBr \text{ (grams)} = \text{plasma volume (milliliters)} \times (1.063 - 1.019) \times 0.94 \text{ (constant)}/(1 - (0.295 \times 1.063))$$

which was centrifuged against the density gradient for 18 hours at 40,000 rpm at 10°C. The supernatant ($d=1.019 - 1.063$ g/ml) contained the LDL fraction. HDLs ($d=1.063 - 1.21$ g/ml) were isolated by further sequential ultracentrifugation (43,000 rpm for 44 hours at 10°C; potassium bromide $d=1.210$ g/ml) according to the formula

$$KBr \text{ (grams)} = \text{plasma volume (milliliters)} \times (1.210 - 1.063) \times 0.94 \text{ (constant)}/(1 - (0.297 - 1.210))$$

The lipoproteins were dialyzed against phosphate-buffered saline (PBS), pH 7.4, containing 0.1 mg/ml EDTA and stored at 4°C for no longer than 1 week. The total protein content of the lipoproteins was analyzed by the method of Lowry et al\textsuperscript{27} and was used to determine HDL amounts for labeling after ultrafiltration through Centrissart UF membranes (Sartorius, Göttingen, FRG). The apoprotein composition of each of the lipoprotein classes was assessed by radial immunodiffusion.

**Radiolabeling**

HDLs were labeled with $^{111}$In according to the methodology described recently for LDL labeling.\textsuperscript{28} For each series of experiments, the lipoproteins of one normolipemic subject were used. To a microvial equipped with a magnetic stirrer, 4 mg HDL in 200 µl PBS (pH 7.5), 20 µl 0.5 M NaHCO$_3$ and 36 µg cyclic diethylenetriaminepentaaacetic acid (DTPA) anhydride/mg lipoprotein (Sigma Chemical Co., St. Louis, Mo.) in 9 µl dry dimethyl sulfoxide (Merck, Darmstadt, FRG) were added. The mixture was slowly stirred for 1 hour and applied to a 5 x 40-mm Sephadex G50F column (Pharmacia, Uppsala, Sweden) equilibrated in metal-free acetate-buffered saline (ABS), pH 5.5. The column was eluted with ABS and the protein fraction (240 µl) collected into a microvial. To this mixture, 600 µCi $^{111}$InCl$_3$ in 40 µl 0.04 M HCl was added with gentle mixing. After 1 hour at room temperature the reaction mixture was applied onto a second ABS-equilibrated Sephadex G50F column. The $^{111}$In-labeled protein fraction (350 µl) was collected and mixed with 1 mM DTPA in PBS to give 1 ml of the final product solution.

Radiochemical purity was determined by 1) thin-layer chromatography (TLC) with silica gel plates (Merck) and an eluant containing 10% (vol/vol) ammonium formate, and 0.5 M citric acid (20:20:10; vol/vol/vol); 2) cellulose acetate electrophoresis with 0.05 M barbital buffer, pH 8.6, containing 1 mM EDTA and 1% human serum albumin at 300 V for 20 minutes; and 3) polyacrylamide gel electrophoresis (PAGE) with gradient gels (T=8–18%) and a gel buffer of 0.12 M Tris(hydroxymethyl)aminomethane (Tris), 0.12 M acetate, and 0.1% sodium dodecyl sulfate, pH 6.4 (LKB/Pharmacia, Uppsala, Sweden), 50 mM/gel, 200–600 V for 60 minutes.

For selected experiments HDLs were also labeled with $^{125}$I as described recently for LDL labeling.\textsuperscript{29} Briefly, in a microvial 500 µl chloroform solution containing 30 µg Iodogen was evaporated with a stream of nitrogen, redissolved, and blown dry again to produce a homogeneous surface coating. To the Iodogen-coated vial, 1 mg HDL in PBS, pH 7.5, 0.15 µg NaI carrier, and approximately 0.2 mCi $^{125}$I-NaI were added. The reaction mixture (500 µl) was stirred slowly at 4°C for 10 minutes and applied to a dialysis bag containing dialysis buffer (0.15 M NaCl, 0.01 M phosphate [pH 7.5], and 0.2 mM EDTA) at 4°C. Radiochemical purity was routinely determined by trichloroacetic acid (TCA) precipitation and cellulose acetate electrophoresis. $^{125}$I-HDL was also analyzed by PAGE and high-performance liquid chromatography size-exclusion chromatography.

**Binding Studies**

**Patients and healthy controls.** Thirty-six patients (14 men and 22 women aged 19–62 years) with heterozygous FH and 15 healthy normolipemic volunteers (eight men and seven women aged 22–49 years) were studied.

All normolipemic control subjects had not taken medication for at least 3 weeks. None of the control subjects was a smoker or had any other known risk factor for the development of atherosclerosis. To examine the individual variation of the binding data, eight healthy volunteers were investigated twice for HDL receptor expression on platelets. The second investigation was performed within 3 months.

FH patients taking drugs known to interfere with platelet function, lipid metabolism, or the prostaglandin
system were excluded from the study. All patients were confirmed to be on a low-cholesterol diet for at least the previous 6 months.

The effect of a 2-month “controlled diet period” on HDL receptor expression was studied in eight FH patients. For this period each patient received a diet plan calculated for a daily cholesterol intake of <300 mg. Furthermore, the patients were required to complete a questionnaire that assessed their definite daily consumption in terms of eating and drinking. The questionnaire was controlled and calculated for total daily cholesterol intake by a trained dietary assistant.

To study the effect of a lipid-lowering medication in 10 patients, HDL receptors were estimated before, after 2 months, and after 6 months of treatment with the clonifibrate analogue gemfibrozil, alone (450–900 mg daily) or in combination with cholestryramine (8–16 g daily).

Platelet isolation and characterization. All blood processing was carried out in plastic ware at room temperature. In the morning after an overnight fast, venous blood (60 ml) was collected through siliconized needles into 3x20-ml plastic syringes containing acid-citrate-dextrose (3:7, vol/vol). In preliminary experiments platelets were isolated by differential centrifugation. Platelet-rich plasma was prepared by centrifugation (150g for 10 minutes). In a further centrifugation step (1,500g for 25 minutes), the platelet pellet obtained was washed twice in the following solution: isotonic NaCl solution (20 ml), 50 mM Tris hydrochloride buffer, pH 7.4 (2 ml), and 2% NaEDTA (100 µg/ml) neutralized with sodium hydroxide (2 ml), with pH adjusted to 7.2. The washed pellet was taken up in assay buffer containing 50 mM Tris hydrochloride, pH 7.5, 5 mM CaCl₂, and 5 mM MgCl₂. Platelet counts were determined electronically and by counter flow.

The effectiveness of the washing procedure in removing autologous plasma proteins was assessed by adding 111-In-HDL to the platelet-rich plasma. Based on the recovery of radioactivity in the isolated platelet suspension, 8±2 ng HDL was present per 10⁶ platelets.

The interaction of 111-In-labeled HDL with washed human platelets was assessed after centrifuging the platelets to separate bound from free ligand. As judged by electron microscopy, the separation method did not induce cell lysis or considerable platelet activation. Platelet-specific proteins (β-thromboglobulin and platelet factor 4 concentrations) as well as RTLC-measured [¹⁴C]arachidonic acid metabolites were not significantly (quantitatively and qualitatively) altered by the procedure (I. Virgolini et al, unpublished observations).

Platelet aggregation. Platelet aggregation was studied in a Born-type aggregometer.²⁸ Platelets (2.5x10⁴/µl in 600 µl) were aggregated with 1 µM ADP (100 µµ). Prostaglandin (PG) I₂ (The Upjohn Company, Kalamazoo, Mich.) was used as an indicator for inhibition of platelet aggregation. The effect of LDL and HDL on platelet aggregation was determined by addition of various concentrations of the labeled and unlabeled lipoproteins 10 minutes before induction of aggregation with ADP. The aggregation response was verified by the height and slope of the response curve.

Binding Assays

To investigate ligand binding to the HDL receptor of platelets, direct binding experiments were carried out. The conditions of the assay system were essentially the same as reported for 111-In-LDL binding to human liver.²⁸ All lipoprotein fractions (labeled and unlabeled) were assessed spectrophotometrically for their protein content by dye binding by using the assay kit provided by Bio-Rad Laboratories (Coomassie Blue Reagent G20; Richmond, Calif.).

Incubations were done in duplicate. The within-assay variability amounted to 3.8±1.0% and the between-assay variability to 6.7±1.1%.

In the initial experiments, the time course of association in binding was studied by incubating the platelets (10⁶ cells) with 111-In-HDL (5 µg protein/ml) in the absence (total binding) or presence (nonspecific binding) of unlabeled HDL (200 µg protein/ml) for 1–120 minutes. Dissociation of binding was induced by the addition of excess unlabeled HDL (200 µg protein/ml) at different time intervals (1–60 minutes) at equilibrium.

The influence of temperature on binding was studied through equilibrium experiments carried out at 4°C, 15°C, 22°C, and 37°C.

In competition experiments, the cells were incubated at room temperature for 45 minutes with 5 µg protein/ml 111-In-HDL (925 cpm/ng protein) in the absence or presence of increasing concentrations (0.1–500 µg protein/ml) of unlabeled HDL or unlabeled LDL, respectively. To evaluate the specificity of 111-In-HDL binding onto platelets, several unrelated proteins (albumin, fibrinogen, myoglobin, ovalbumin, soybean trypsin inhibitor, and transferrin) were also tested for their capacity to displace bound 111-In-HDL using a 25- to 50-fold molar excess of each unlabeled competitor protein.

In saturation experiments, the cells were incubated with increasing concentrations of 111-In-HDL (0.1–70 µg protein/ml) in the absence (total binding) or presence (nonspecific binding) of unlabeled HDL (200 µg protein/ml). Specific binding (SB) was determined as the difference between total and nonspecific binding.

After incubation, the tubes were rapidly centrifuged (1,500g, 10 minutes, 4°C) to separate free from membrane-bound ligand. After it was washed, the pellet was counted in a gamma counter for 1 minute.

In the absence of platelets, the application of 30 µg protein of 111-In-HDL resulted in the recovery of <1 µg protein of 111-In-HDL/ml in the tip of the tube after centrifugation (<4%). This amount was identical for incubations for total (TB) and nonspecific (NSB) binding.

In typical experiments, nonspecific binding amounted to less than 8% of total binding [SB=TB−NSB=100−(<10%±90%)]

Analysis

Binding data were calculated according to Scatchard.²⁰ Values are presented as mean±SD. Statistical analysis was done by standard statistical tests, including Student’s t test, analysis of variance, and simple linear-regression analysis at a confidence level of 95%.

Results

Human plasma HDLs (d=1.063–1.21 g/ml) were isolated by density gradient ultracentrifugation in potassium bromide and conjugated with DTPA chelator by
reacting the bicyclic DTPA anhydride with HDL in a molar ratio of 10:1 (apo A-I, A-II, and C-II, with an average molecular weight of 100 kd). At this molar ratio of reactants, the average number of DTPA groups bound per HDL molecule amounted to 2.3 (range, 1–4). The lipoprotein–DTPA conjugate was labeled with \(^{111}\text{In} \) in isolated radiochemical yields of 72±9\% (range, 55–85\%). This resulted in a specific activity of 120–160 \(\mu\text{Ci/mg protein}\). The labeled products were routinely analyzed by TLC (Figure 1) and cellulose acetate electrophoresis (Figure 2). The clearly separated un conjugated \(^{111}\text{In-DTPA}\) and free \(^{111}\text{In}^{3+}\) ions contained <1\% total radioactivity. On gradient PAGE, \(^{123}\text{I-HDL} \) and \(^{111}\text{In-DTPA-HDL}\) displayed identical bands (silver staining) compared with the unlabeled lipoprotein.

To determine the percentage of total radioactivity bound to the product, aliquots of \(^{111}\text{In-HDL}\) were added to unlabeled lipoprotein and analyzed by ultracentrifugation at a potassium bromide density of 1.21 g/ml. The radioactivity found amounted to >96±2\% in the lipoprotein–DTPA-HDL. Ninety-six percent of the radioactivity of \(^{111}\text{In-HDL}\) was precipitated with 10\% (final concentration) TCA. Further evidence for the retention of natural biological activity of \(^{111}\text{In-HDL}\) was obtained in aggregation experiments with washed human platelets. As isolated, the platelets exhibited typical aggregation responses to ADP and normal sensitivity to PGI\(_2\). Unlabeled HDL and \(^{111}\text{In-HDL}\) caused significant inhibition of ADP-induced platelet aggregation, with a half-maximal inhibitory dose at half-maximal association (IC\(_{50}\)) of 10.2±3.1 and 11.3±3.2 \(\mu\text{g protein/ml}\), respectively, whereas labeled and unlabeled LDL significantly enhanced aggregation behavior, with median effective dose (ED\(_{50}\)) values of 33.2±5.1 and 31.3±6.1 \(\mu\text{g protein/ml}\), respectively.

The ability of \(^{111}\text{In-HDL}\) to competitively inhibit binding of \(^{123}\text{I-HDL}\) to specific receptors was compared with that of unlabeled HDL (Figure 3). \(^{111}\text{In-HDL}\) significantly inhibited binding of \(^{123}\text{I-HDL}\) (5 \(\mu\text{g protein/mL}\) to the same extent as unlabeled HDL (IC\(_{50}\) was 8 and 6 \(\mu\text{g protein/mL}\) for \(^{111}\text{In-HDL}\) and unlabeled HDL, respectively), demonstrating its equivalence with \(^{123}\text{I-labeled HDL and native HDL with respect to reactivity with platelets.}

Incubations of \(^{111}\text{In-HDL}\) prepared to different specific activities (with unlabeled HDL) with platelets, while maintaining a constant total HDL concentration of 10 \(\mu\text{g protein/mL}\), identified the linear relation of platelet-bound \(^{111}\text{In-HDL}\) to the percentage of \(^{111}\text{In-HDL}\) present in the incubation mixture (Figure 4). This reflects the same apparent affinity of \(^{111}\text{In-HDL}\) and unlabeled HDL for platelets.
In initial binding studies, the interaction of $^{111}$In-HDL with washed human platelets was assessed as a function of time and temperature. As shown in Figure 5, $^{111}$In-HDL bound to the washed platelets at 22°C, and the time course of the binding reaction shows a rapid increase of binding for approximately 5 minutes and the apparent attainment of equilibrium at 25 minutes. In the same experiment after a 60-minute incubation, displacement of $^{111}$In-HDL by excess unlabeled HDL (200 μg protein/ml) was achieved. This indicates that 90% of the bound material is not incorporated under the assay conditions maintained.

The interactions were only slightly temperature dependent. At 22°C specific binding of $^{111}$In-HDL at 45 minutes was 96±5% of that observed at 37°C, at 15°C it was 93±7%, and at 4°C it was 90±6%. Whereas total binding increased slightly, nonspecific binding did not change when the incubation temperature was increased. In all subsequent experiments, lipoprotein binding was measured at 22°C, and we chose a 45-minute incubation time to ensure attainment of equilibrium.

Unlabeled HDL as well as LDL caused significant inhibition of $^{111}$In-HDL binding to washed human platelets (Figure 6). The corresponding IC₅₀ values for unlabeled HDL were significantly ($p<0.001$) lower for normolipemic volunteers (Table 1, $n=15$; 14±3 μg protein/ml) compared with the FH patients (Table 2, $n=34$; 20±4 μg protein/ml).

Figure 3. Binding competition curves showing ability of $^{111}$In-high density lipoprotein (HDL) and unlabeled HDL of normolipemic subjects to compete with $^{125}$I-HDL for binding to washed human platelets. Each assay tube contained human $^{125}$I-HDL (625 cpm/ng protein) and the indicated concentrations of $^{111}$In-HDL or unlabeled HDL. The 100% control value for $^{125}$I-HDL binding was 1,032±91 ng protein/10⁹ platelets. Each point represents the mean of six independent determinations with platelets from different subjects.

Figure 5. Time course of specific $^{111}$In–high density lipoprotein (HDL) binding to washed human platelets. For association, $^{111}$In-HDL (5 μg protein/ml) was incubated with 10⁹ platelets in the absence (total binding) or presence (nonspecific binding) of unlabeled HDL (200 μg protein/ml) for the time intervals indicated. For dissociation, to study the time course of displacement of specific binding, excess unlabeled HDL (200 μg protein/ml) was added at equilibrium (i.e., after 60-minute incubation). Each point represents the mean±SD of three independent experiments with washed platelets derived from different volunteers.
As listed in Table 1, no significant difference was found for men and women. As listed in Table 1, no significant difference was found for the mean values of the first and second evaluations. With the exception of volunteer No. 10, in all volunteers an acceptable variation (about 5-10%) in ¹¹¹In-HDL binding onto platelets was found.

A group of 36 patients with FH and various other risk factors was investigated for platelet binding of "normal" ¹¹¹In-HDL. As listed in Table 2, there was remarkably high variation in the ¹¹¹In-HDL binding data. Whereas some patients had values that were almost in the normal range, others had amazingly low HDL binding capacities. On average, the ¹¹¹In-HDL binding capacity amounted to 1,012±439 ng protein/10⁹ platelets (i.e., 6,072±2,635 sites/platelet; n=34; two patients with alcohol anamnesis were excluded from the statistical evaluation), which was significantly (p<0.01) lower pared with that of normolipemic control subjects. Furthermore, K₅ (12±4 μg protein/ml; p<0.01) and IC₅₀ (22±9 μg protein/ml; p<0.001) values were significantly higher in FH patients compared with normolipemic volunteers. The binding of ¹¹¹In-HDL onto platelets of one FH patient with a low binding capacity is shown in Figure 8.

A significant correlation was found between ¹¹¹In-HDL binding data and plasmatic lipid and lipoprotein concentration (Table 3). The linear-regression analyses show that the higher the total plasmatic cholesterol, LDL cholesterol, and apo B concentrations and the lower the HDL cholesterol and apo A-I concentrations, the lower are the number of HDL binding sites on human platelets and the higher are the K₅ and IC₅₀ values.

No correlation of age (Table 3) with ¹¹¹In-HDL binding to platelets was found. The heterogeneous group of FH patients was divided into several subgroups (Table 4) to explore the higher variation observed among the FH patients investigated. No significant difference was found for men and women. Those without other risk factors in addition to hyperlipidemia had a significantly (p<0.05) higher HDL binding capacity than those with at least one additional risk factor. Smokers showed significantly (p<0.05) lower values than nonsmokers, and those patients who smoked and who had manifested atherosclerosis in terms of coronary heart disease (CHD) and/or peripheral vascular disease (PVD) had an even lower HDL binding capacity (p<0.01). Hypertensive subjects showed a lower value than normotensive subjects (p<0.01), and those who were smokers and hypertensive had the lowest values (p<0.01) of all.

No difference was observed for diabetics and nondiabetics or the groups that were matched according to the presence or absence of CHD and/or PVD. The effect of a 2-month "controlled diet period" on HDL receptor expression was studied in eight FH patients (Table 5). With the exception of one patient (No. 20, Table 5), a small increasing effect on HDL...
receptor density of platelets was observed; however, no significance was found for the mean values.

Ten of the patients listed in Table 2 were treated with lipid-lowering drugs (gemfibrozil, alone or in combination with cholestyramine) to investigate changes in platelet HDL receptor activity. The binding results (Table 6) were well correlated with lowered plasmatic total cholesterol, LDL cholesterol, and apo B concentrations as well as with increased HDL cholesterol and apo A-I concentrations after a 2-month and 6-month treatment period. In some patients, treatment raised apo A-I concentrations after a 2-month and 6-month treatment period. In others, treatment raised apo A-I concentrations but not other lipoprotein concentrations. Maximal binding capacity (Bmax) is in nanograms of protein of In-HDL bound per 10^9 platelets; dissociation constant (Kd) is in micrograms of protein per milliliter.

### Discussion

So far, only a few studies have investigated the interaction of HDL with platelets. The results are, however, largely conflicting with respect to the presumptive ability of HDL to affect the activation stage of platelets. Depending on the parameter measured as well as the HDL fraction applied, both platelet activating and deactivating processes were reported, e.g., granule centralization by gold-labeled HDL and enhanced ADP-induced aggregation by high concentrations of HDL on the one hand but diminished platelet aggregation by apo E-rich HDL and diminished thromboxane B2 production, or an influence counteracting the effects induced by LDL on the other hand. These results suggest a direct interaction of HDL with platelets. Although specific HDL binding to platelets derived from healthy individuals has been shown, the unconditional acceptance of a true physiological receptor has met with some resistance. Here, we provide further evidence for specific HDL binding sites on human platelets in normolipidemia and hyperlipidemia. These sites bind LDL also but not other unrelated proteins.

The possible function of HDL binding to platelets has so far been discussed only in relation to the effects of LDL on platelet function, for which numerous in vivo and in vitro studies report an activation of platelets by high LDL concentrations. The binding of HDL to specific binding sites of the platelet surface membrane most likely does not serve for reverse cholesterol transport for two reasons. First, there is no cholesterol...
biosynthesis in platelets, and they are unable to take up significant amounts of cholesterol once released from the megakaryocytes. Thus, synthesis of cholesterol by megakaryocytes may be the principal source for the cholesterol content of platelets.\textsuperscript{38} This is why under physiological conditions there is no need for reverse cholesterol transport in platelets. Second, it has been shown for several cell types that HDL receptors involved in reverse cholesterol transport are upregulated by an excessive supply of cholesterol.\textsuperscript{39} Our ex vivo results with platelets show the opposite effect with respect to the influence of plasmatic cholesterol levels on the number of HDL binding sites. In the hypercholesterolemic state, the HDL binding capacity was diminished, and after treatment with lipid-lowering drugs, a decrease in plasmatic cholesterol was associated with an increase in HDL binding sites that approached the values measured in healthy normolipemic control subjects. Even though the influence of high cholesterol concentrations on platelets should take place at the level of megakaryocytes and therefore is not unequivocally comparable with the in vitro results cited above,
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"In-HDL bound (ng protein/10^9 platelets)

FIGURE 7. Saturation curve (left panel) and corresponding Scatchard plot (right panel) of specific 111In–high density lipoprotein (HDL) binding to washed human platelets from a normolipemic volunteer (No. 8; Table 1). Each assay tube contained the indicated concentrations of 111In-HDL (925 cpm/ng protein). Specific binding was calculated by subtracting the amount of 111In-HDL bound in the presence of excess unlabeled HDL (200 ng protein/ml; nonspecific binding) from that bound in its absence (total binding). Scatchard analysis indicated a single saturable binding site capable of binding 1,698 ng protein of 111In-HDL/10^9 platelets specifically. Corresponding dissociation constant was 6 μg protein/ml.

In this study we compared the specific binding of 111In-labeled HDL to platelets in hyperlipemia and normolipemia, from which we might gain some insights into the significance of HDL binding to platelets in vivo. To our knowledge such a study has not been approached before. As a radiolabel we used 111In-HDL because in vivo studies with 111In-labeled lipoproteins may have some advantages over radioiodinated lipoproteins.29,30 Competition experiments with 111In-HDL, 123I-HDL, and unlabeled HDL demonstrated virtually identical binding behavior of the two differently labeled species and native HDL. As shown by TLC, cellulose acetate electrophoresis, repeated ultracentrifugation, and TCA precipitation, 111In-HDL fulfills the requirement of radioactive purity for receptor studies. Furthermore, aggregation experiments with platelets from healthy subjects showed that 111In-HDL retained its biological activity. We measured the same apparent affinity of unlabeled and 111In-labeled HDL for platelets.

Equilibrium binding of 111In-HDL reached half-maximal saturation at 7 μg protein/ml HDL, giving a K_d of 7×10^-8 M (7±3 μg protein/ml) and 1,882 ng HDL protein/10^9 platelets bound, corresponding to 11,292 binding sites per platelet. The small influence of temperature on binding and the rapid association of binding are in good accordance with our previous results with 125I-HDL.32 In this ex vivo comparison of binding parameters in healthy subjects and FH patients, in contrast to our previous study we used the whole-plasma HDL fraction (d=1.063-1.21 g/ml) and modified the platelet isolation and washing procedures. Therefore, the quantitative values for K_d and the number of apparent binding sites differ from our values obtained with HDLd. The whole HDL fraction comprises a heterogeneous population of particles that can be separated into two major density classes, HDL1 and HDL2, and which contain further subgroups with respect to chemical composition and their established and putative in vivo functions. If only some distinct subclass(es) of HDL particles are able to bind to platelets, then the measured binding parameters will depend on the relative concen-
The same method that was used to obtain the binding parameters with $^{111}$In-HDL and platelets from healthy subjects was applied to platelets from FH patients as listed in Tables 2–6. The binding parameters for the patients show a significantly ($p<0.01$) lower number of binding sites for $^{111}$In-HDL compared with those obtained for the healthy normolipemic volunteers. Additionally, significantly higher $K_d$ ($p<0.01$) and $IC_{50}$ ($p<0.001$) values were found with platelets from FH patients, indicating weaker binding of HDL to this lower number of binding sites.

The greater variation in binding parameters within the group of patients might be a consequence of the fact that they were very heterogeneous with respect to the presence or absence of additional risk factors (i.e., smoking, hypertension, and diabetes) and CHD/PVD, sex, and age. In a more detailed analysis (Table 4), we subgrouped the patients collectively and obtained very interesting results. Whereas HDL receptor expression was obviously not dependent on age, sex, or the presence of CHD/PVD, those patients without additional risk factors had higher HDL receptor numbers than those with at least one risk factor in addition to hyperlipidemia. The lowest number of HDL binding sites was found for FH patients who were smokers and who had hypertension. It is widely recognized that smoking enhances platelet reactivity and may lower the plasmatic level of HDL. Direct association of HDL with hypertension has not yet been reported. Hypertension may increase the incidence of atherosclerotic disease, probably by increasing the permeability of the endothelium and thereby leading to an increased uptake of cholesterol and platelet constituents, which in turn promotes smooth muscle cell proliferation. Although there is evidence that platelet reactivity is enhanced in diabetes mellitus and that platelet microthrombi have been observed in diabetic microangiopathy, no difference has been observed between diabetics and nondiabetics at the HDL receptor level of platelets.

The underlying mechanisms regulating the number of HDL binding sites on platelets are not clear. Control experiments with autologous lipoproteins (not shown here) support the interpretation that platelets from FH patients differ in their HDL receptor status from those from healthy donors and can be reverted to that status on treatment of the hyperlipidemia. Principaliy, hyperlipidemic states can influence the development of megakaryocytes, leading to abnormal platelets. Whether or how much the presence of abnormal platelets contributes to reduced HDL binding in FH cannot be assessed at the moment. Second, the alterations might occur at the platelet level, leading to an altered arrangement and/or localization of the binding protein in the plasma membrane.

We have identified the proteins responsible for binding of HDL in the platelet membrane as the glycoproteins GPIIb and GPIIIa. These glycoproteins exist as a calcium-dependent complex and are well known as fibrinogen receptors, playing a central role in the early stages of platelet activation. Obviously even in normal states only a minor fraction of the total GPIIb/IIIa is capable of strongly binding to HDL. This fraction appears to be further decreased under the conditions of FH, probably by either rearrangement within the plasma membrane or redistribution of membrane glycoproteins between different...
Values are mean±SD.

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| Normal values for cholesterol (cholesterol) are <200; for triglycerides (triglycerides), <150; for high density lipoprotein cholesterol (HDL), >50; for low density lipoprotein cholesterol (LDL), <13; for apolipoprotein (apo) A-I, >100; and for apo B, <130 mg/dL. Maximal binding capacity (Bmax) is in nanograms of protein of 131In-HDL bound per 10^9 platelets; dissociation constant (Kd) and half-maximal inhibitory doses at half-maximal association (IC50) are in micrograms of protein per milliliter. “a” and “b” are independent determinations within 3 months.

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This cross-competition raises the question whether there exist two different (lipoprotein-specific) receptors or only one (lipoprotein-specific) receptor with a “relaxed specificity.” Careful investigation in binding and inhibition studies as well as mathematical evaluation of the experimental data suggest that the inhibitory behavior is a “noncompetitive, mixed or allotopic inhibition” according to the enzyme inhibition nomenclature introduced in 1980 by Halsal. From this investigation it seems possible that two different receptors for LDL and HDL exist on the human platelet, but they obviously interfere with one another to some degree. Hence, the binding of HDL and LDL to the same glycoprotein should take place at different sites of the large glycoprotein complex, thereby influencing the structure of the complex in such a way that the binding affinity is lowered for each lipoprotein class in the presence (binding) of the other one. This problem of specificity has already been addressed by other authors as well.

The early stages of platelet activation are not yet understood, but it is known that binding of fibrinogen to GPIIb/IIIa is a prerequisite of aggregation. Therefore, the various well-documented modulating effects of LDL and HDL on the platelet activation process might be directly linked to this binding.

It is not clear whether the binding of LDL and HDL to GPIIb/IIIa contributes to the activation procedure or interferes with it and to what extent they act this way. However, it seems possible that the net effect of the lipoproteins in vivo depends on the relative rather than absolute amounts of platelet-stimulating LDL and the platelet-deactivating subtraction of HDL, respectively, bound to their common binding proteins. Diminished binding capacities for both lipoproteins might disturb the balance of the proactivating and antiactivating influences of the different lipoprotein species.

The correlation of low HDL receptor numbers on platelets with hyperlipidemia and the reversibility of the
decrease after lipid-lowering medications support the hypothesis that high-affinity HDL binding is involved in the alterations that take place in hyperlipidemic disorders, possibly because of platelet reactivity.

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Binding of 111In-labeled HDL to platelets from normolipemic volunteers and patients with heterozygous familial hypercholesterolemia.
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