A flow-cytometric method with fluorescence-labeled monoclonal antibodies (MABs) against the low density lipoprotein (LDL) receptor (C7A MAB) or 3,3’-dioctadecylindocarbocyanin-iodide (Dil) LDL has been developed that allows the quantification of LDL receptors on leukocytes and the identification of patients with familial hypercholesterolemia (FH) within 48 hours. Leukocytes were isolated from 10 mL anticoagulated blood by density gradient centrifugation. To induce maximal expression of LDL receptors, mononuclear cells were preincubated with either phytohemagglutinin (PHA) or lipoprotein-deficient serum (LPDS). LPDS-treated monocytes provided a more homogeneous cell population with regard to LDL receptor activity than did the PHA-treated lymphocytes; they also provided a greater discrimination between the fluorescence of the receptor probes and cellular autofluorescence. The C7A MAB was able to compete for Dil LDL binding by about 40%. In competition with unlabeled LDL, Dil LDL revealed linear binding, indicating an affinity similar to native LDL. The binding characteristics of Dil LDL were also similar to 125I-LDL binding. LDL isolated from familial defective apolipoprotein B-100 was not able to compete for Dil LDL binding on monocytes, whereas native LDL reduced it by about 80%. In monocytes from FH heterozygous patients, the cellular mean fluorescence using either C7A MAB or Dil LDL at 4°C was 30% to 70%; in FH homozygotes, cellular mean fluorescence was less than 20% of that in monocytes from normal individuals. In patients with familial defective apolipoprotein B-100 antibody binding was normal, but one patient's own LDL failed to compete with normal Dil LDL for 4°C binding on U937 test monocytes. Patient monocytes having internalization defects showed normal 4°C Dil LDL binding, but at 20°C cell-associated fluorescence was reduced by about 40%. In our study 384 hypercholesterolemic patients (preselected according to serum cholesterol levels, clinical symptoms, and family history) were analyzed for LDL receptor expression using the C7A MAB-based assay. In 71.8% of the patients with cholesterol levels higher than 300 mg/dL, an LDL receptor deficiency was observed. Apolipoprotein E isoforms and lipoprotein[a] were found to be independent from the LDL receptor status. In some patients with high cholesterol levels but normal LDL receptor expression with the C7A MAB assay, LDL receptor defects could be diagnosed when either reduced binding or internalization of Dil LDL or familial defective apolipoprotein B-100 was detected. We conclude that fluorescence flow cytometry provides an appropriate, easily performed assay system for the differential diagnosis of LDL receptor defects, including LDL receptor deficiencies and internalization defects, and also allows the discovery of ligand defects.

Key Words • LDL receptor analysis • familial hypercholesterolemia • fluorescence flow cytometry • monocytes • lymphocytes

On the basis of epidemiological and clinical studies the direct role of cholesterol in the development of atherosclerosis and coronary heart disease has been clearly established. More specifically, familial hypercholesterolemia (FH) is genetically and biochemically well defined. Heterozygotes with FH occur at a frequency of about 1 in 500, whereas homozygotes constitute 1 in 100,000. The disorder results from the genetic defect in the cell surface low density lipoprotein (LDL) receptor that normally controls the degradation of LDL, the major transport protein for cholesterol in human plasma. The molecular bases of approximately 40 mutations in the LDL receptor gene on chromosome 19 have been defined. There are four biochemical classes of mutations. These include defects in LDL receptor synthesis, transport, ligand binding, and internalization. The clinical manifestation of FH is characterized by three cardinal features: selective elevation in the plasma level of LDL; deposition of LDL-derived cholesterol in tendons and arteries, forming atheromas; and inheritance of an autosomal dominant trait with a gene-dosage effect. The diagnosis of FH in clinical practice is mostly based on serum total cholesterol, LDL cholesterol (LDL-C), high density lipopro-
tein cholesterol (HDL-C), and triglyceride determinations. In some cases the heterozygous form of FH cannot be unequivocally differentiated from elevated serum lipid and lipoprotein values alone because of an overlap with other types of hypercholesterolemia.

Besides FH, a variety of processes under genetic control also lead to enhanced plasma cholesterol levels, such as apolipoprotein (apo) E polymorphism, increased synthesis of lipoprotein[a] (Lp[a]), apoB-100 binding defects, and acid lipase deficiency. Therefore, it is important to have appropriate methods for the differential diagnosis of the causes of hypercholesterolemia. Among the apoE isoforms, apoE4 is associated with increased LDL-C levels, whereas apoE2 is associated with relatively low levels. The reason for this difference lies in the relative affinities of the isoforms to the LDL receptor. ApoE4 has the highest turnover; very low density lipoprotein (VLDL) containing apoE4, therefore, is quickly cleared from the circulation and downregulates the expression of the apoB/E receptors, especially in the liver.

Lp[a], a variant of LDL, is an independent risk factor for atherosclerosis. It consists of apo[a] linked by a disulfide bridge to apoB-100. Apo[a] has several isoforms; the amount of these in the circulation is strongly genetically determined, and levels greater than 25 mg/dL are highly atherogenic. It interacts with the apoB/E receptor, although it is a poor ligand. Apo[a], because of its similar structure to plasminogen, competes with it for the plasminogen binding sites, thereby suppressing endothelial cell fibrinolysis and producing a procoagulant state.

It has recently been suggested that the substitution of glutamine for arginine at residue 3500 of apoB-100, called familial defective apo B-100 (FDB), an autosomal dominantly inherited disorder, disrupts the conformation of the receptor binding domain, leading to increased serum cholesterol levels and premature atherosclerosis. Acid lipase deficiency is another genetic cause of abnormal cholesterol metabolism. The enzyme catalyzes the lysosomal degradation of cholesterol esters. The enzyme deficiency leads to one of two diseases: Wolman's disease or cholesteryl ester storage disease.

Defects in lipoprotein metabolism are often combined in a single individual. On the other hand, dietary factors also undoubtedly contribute to high cholesterol levels. Fox et al showed that the well-known increase in LDL-C in baboons on diets high in saturated fatty acids is related to a reduction of mRNA for the LDL receptor. Serum lipid levels are also influenced by hormonal factors. LDL concentration increases with the decline in estrogen levels in postmenopausal women. There is experimental evidence that the synthesis of apoB/E receptors in hepatocytes is stimulated by estrogen via the pituitary axis, and it is suggested that estrogen may be a major hormonal determinant of receptor expression in fasted middle-aged and elderly women. Other secondary hypercholesterolemias of endocrine origin are seen in hypothyroidism and diabetes. In diabetes the increased triglyceride, VLDL, and LDL levels are the consequences of the lack of insulin action on hepatic lipases and the formation of remnant or glycosylated lipoproteins.

Worldwide efforts are underway to detect and treat patients with high serum cholesterol levels. The prospects of FH gene therapy have even been discussed. The management of medical conditions depends on an understanding of their pathogenesis. Therefore, the differential diagnosis of hypercholesterolemia based on LDL receptor activity is important for therapy. Until now, LDL receptor determinations have been predominantly used in research laboratories. Most of the LDL receptor studies are based on the classic experiments of Goldstein and colleagues with 125I-labeled LDL binding and degradation of human fibroblasts and lymphocytes. There were also attempts to determine LDL receptor activity by changes in cellular functions such as lymphocyte proliferation.

Materials

Blood (10 mL) was collected into monovettes containing 1.5 mg K2-EDTA per milliliter (Sarstedt, Nümbrecht, FRG). LeucoPREP tubes were from Becton Dickinson (Heidelberg, FRG), RPMI-1640 and phosphate-buffered saline (PBS) were from Gibco (Egggenstein), and autologous human serum was collected from the patients to be analyzed. Lipoprotein-deficient serum (LPDS) was prepared by ultracentrifugation by using the dialyzed 1.21 g/mL bottom. For the detection of defective ligands, U937 test monocytes were used. The MAB against the LDL receptor was purified from the culture medium of the C7A monoclonal antibody (MAB) against the LDL receptor into culture medium of the C7A cell line as described previously. Alternatively, the LDL receptor MAB was purchased from Amersham (Braunschweig, FRG) and was directly labeled with FITC using the method of Kawamura.

The FITC-labeled second antibody (FITC goat anti-mouse [GAM]) was from Becton Dickinson, and the tissue culture equipment was from Falcon (Heidelberg), except the culture flasks, which were from Greiner (Frickenhausen, FRG). RITC and bovine serum albumin (BSA) were purchased from Serva (Heidelberg), 3,3′diodotyrosilcocarbocyanin-iodide (DiI) from Molecular Probes (Eugene), IODO-beads from Pierce Chemical Co (Frankfurt, FRG), and fetal calf serum from Boehringer (Mannheim, FRG).
Patients

Normocholesterolemic control subjects and hypercholesterolemic patients (plasma cholesterol levels >250 mg/dL) were selected from the clinics of the Westfälische Wilhelms University of Münster during the period from 1988 to 1991. The criteria for involvement in our screening program was a lack of systemic disorders or acute infection. The mean age of patients was 34 years and the ratio of women to men was 0.95. The clinical diagnosis of FH was based on isolated hypercholesterolemia among first-degree relatives. The presence of either xanthomas, xanthelasmas, or an arcur lipoides cornea before the age of 35 years was taken as additional evidence for FH. In the case of LDL receptor defects, especially in cases of homozygous forms, the family members were also screened. All the patients and control subjects were informed about the purpose and use of the investigations. In all patients investigated, lipids (total cholesterol, LDL-C, HDL-C, and triglycerides), LDL receptor activity, Lp[a] levels, apoB-100 mutation to arginine at residue 3500, and the apoE phenotype were routinely determined. Lipids were determined enzymatically by using the Boehringer Mannheim test kit and a Cobas-Bio Centrifugal Analyser (Hoffmann-La Roche, Basel, Switzerland); LDL receptor activity was determined by using flow cytometry as detailed below; Lp[a] levels were determined by using the Bio Rad test kit; and apoE polymorphism was determined enzymatically by using the Boehringer Mannheim test kit and a Cobas-Bio Centrifugal Analyser.

Separation and Preincubation of Monocytes and Lymphocytes

Monocytes were isolated by a modification of the method of Boyum: 10 mL blood containing 1.5 mg K$_2$EDTA per milliliter was layered into LeucoPREP tubes for cell preparation and centrifuged at 800g for 10 minutes to separate the mononuclear cell populations. The mononuclear cell layer was collected with a siliconized Pasteur pipette and washed three times in RPMI-1640 medium. The first washing was centrifuged at 400g and the second and third washings were done at 200g for 10 minutes. After the washing procedure, the mononuclear cells were resuspended in 4.5 mL RPMI-1640, and 500 μL LPDS or human serum was added. The cells were incubated for 48 hours at 37°C in a humidified carbon dioxide incubator in Greiner flasks (12×75 mm). For lymphocyte proliferation, 1 μg/mL phytohemagglutinin (PHA) was added to the culture medium in addition to LPDS, and the cells were incubated for 72 hours at 37°C. U937 monocytes used for competition studies to check defective ligands were also cultured in LPDS for 48 hours as described above.

Labeling of the Cells With the LDL Receptor Antibody

After the incubation time, culture flasks were placed on ice for 60 minutes to reduce the adherence of the monocytes. Cells were replaced from the bottom of the culture flasks with vigorous washing. The mononuclear cells were washed twice with PBS/0.5% BSA in plastic tubes. The adjustment of the cell number was performed by a TOA CC-800 cell counter. Cells (2×10$^6$) were diluted in 1 mL ice-cold PBS/0.5% BSA. Then anti-LDL receptor antibody (0.0 to 0.4 μg/250 μL medium) was added to 200 μL of this cell suspension (4×10$^6$ cells) and incubated for 30 minutes on ice. After the incubation, the samples were washed twice with PBS/0.5% BSA and incubated for 30 minutes in a dilution of 4 μL FITC-labeled anti-mouse IgG (Becton Dickinson) with 16 μL PBS. Then the samples were washed twice with PBS/0.5% BSA. After the final washing, 300 μL ultracount (Becton Dickinson) was added to the samples for flow-cytometric determination.

Fluorescence Flow Cytometry

The measurements were performed on an FACScan (Becton Dickinson) equipped with an argon ion laser and linked to a Hewlett-Packard computer. The 488-nm line of the argon ion laser, run at an output power of 0.2 W, was used for excitation. FITC fluorescence was measured at 520 to 540 nm (FL1) and RITC and Dil fluorescence were measured at 580 nm (FL2). The acquisition number of the cells was set at 10,000. Forward-scatter versus side-scatter gates were set to exclude lymphocytes and dead cells. Fluorescence signals were recorded to produce a histogram of the gated monocytes versus relative fluorescence intensity after logarithmic amplification. The results were expressed as mean fluorescence of the gated monocytes in the FL1 channel. Mean fluorescence values were transformed into a linear scale by the software of the flow cytometer.

To separate dead cells from intact cells, the cells were incubated just before flow-cytometric measurement with 10 μg propidium iodine (PI) in a volume of 50 μL for 15 minutes in the dark at room temperature. Without washing, the cells were directly measured in the FACScan. Dead cells were electronically separated in an FL2 versus volume dotplot. The purity of the monocytes was further checked by a FITC-labeled CD14 MAB (Becton Dickinson). The gated monocytes used for LDL receptor determinations were 99.7% CD14-positive.

Preparation of Lipoproteins

Human LDL (d, 1.019 to 1.063 g/mL) and HDL (d, 1.125 to 1.21 g/mL) were obtained from the plasma of healthy subjects by sequential ultracentrifugation. The lipoproteins were exhaustively dialyzed against a solution containing 0.15 mol/L NaCl and 5 mmol/L EDTA (pH 7.4). For the binding and competition studies, normal LDL and defective LDL from the patients with FDB were isolated with preparative free-flow isochophoresis as previously described. The concentration of lipoproteins was expressed in terms of protein content, determined according to the method of Lowry et al.

Conjugation of LDL With RITC

Two milliliters of the LDL solution (3 to 5 mg protein per milliliter) was diluted with 0.5 mL 0.2 mol/L Na$_2$HPO$_4$. RITC was dissolved in dimethylformamide to give 16 mg/mL. Twenty micrograms RITC per milligram LDL protein was added in small aliquots to the LDL solution with continuous stirring. Then 1 mL 0.1 mol/L Na$_2$HPO$_4$ was added, and the lipoprotein-RITC mixture was adjusted to pH 9.5 by dropwise addition of 0.1 mol/L Na$_2$PO$_4$. Total volume was adjusted to 4 mL with 0.15 mol/L NaCl, and the mixture was incubated with stirring.
in the dark at room temperature for 30 minutes. A PD-10 column (Pharmacia, Freiburg, FRG) was washed with 25 mL PBS and the conjugate was applied to the column, eluted with PBS, and further dialyzed against the NaCl/EDTA buffer overnight.45

Reconstitution of LDL With Dil

Fifty micrograms LDL protein in 5 mL 0.15 mol/L NaCl and 0.3 mol/L EDTA (pH 7.4) was dialyzed against 5 L of 5 mmol/L EDTA (pH 7.0) at 4°C. Aliquots of 200 μL containing 2 mg LDL protein were mixed with 25 mg nonsoluble potato starch in siliconized glass tubes. The samples were lyophilized overnight. Neutral lipids were removed from the LDL-starch complex by threefold extraction with heptane at -20°C. After the last heptane supernate had been removed, the pellet was incubated at -20°C for 1.5 to 2.5 hours with 4.5 mg Dil in 200 μL heptane. The mixture was evaporated under a stream of nitrogen and resuspended in 1 mL of a mixture of 10 mmol/L Tricine at pH 8.2, and each tube was incubated for 48 hours at 4°C. The tubes were then centrifuged for 10 to 15 minutes at 2000 rpm at 4°C. The supernatant containing Dil-reconstituted LDL was centrifuged twice at 12 000 rpm.46 Preparations of LDL were not older than 8 days at the time of the experiments to avoid false-positive binding defects due to the auto-oxidation of LDL.

Characterization of the Fluorescent Lipoprotein Conjugates

The fluorescence/protein (F/P) ratio of the lipoproteins labeled with RITC was determined according to the method of Kawamura.48 The RITC concentration was measured by absorption spectroscopy by using an absorbitivity value (A) of 0.048 per microgram RITC at 550 nm. Protein concentration was determined according to the method of Lowry et al.44 Usually five to eight molecules of RITC bound to one LDL molecule, as calculated assuming relative molecular masses of 536 for RITC and 500 000 for LDL protein. The F/P ratio for the Dil-LDL complex was determined by dissolving a known amount of Dil-LDL protein in ethanol and comparing the absorption with a standard curve for Dil (2 to 10 μg/mL) in ethanol. Usually four to seven Dil molecules bound to one LDL molecule, assuming a relative molecular mass of 931 for Dil. The amount of bound Dil-LDL particles was calculated with the help of high-level quantitative fluorescein microbead standards.

Iodination of LDL

Freshly isolated LDL was iodinated by the IODO-bead method.47 Aliquots of LDL containing 5 to 10 mg LDL protein per milliliter in PBS (pH 7.4) were mixed with 0.5 mCi Na125I. Three to five IODO-beads were added, and the mixture was incubated for 10 minutes at room temperature. Unbound iodine was removed by chromatography on a PD-10 column (Pharmacia) equilibrated with a PBS buffer. The protein fractions were pooled and dialyzed against PBS for 20 hours. Final specific activities were between 150 and 300 cpm per nanogram protein.

125I-LDL Binding on Human Monocytes

For the radioligand assay, cells were washed using the procedure described for the assay using the LDL receptor antibody, except that 1×10⁶ cells were used and total volume was adjusted to 1 mL for the incubation period. The binding assay was performed at 4°C as described by Goldstein et al.28 After counting, cells were digested with NaOH, and their protein content was determined. Specific binding was calculated from total binding minus binding in the presence of excess unlabeled LDL.

Other Methods

Protein determinations were done by using the method of Lowry et al44 using BSA as a standard. To prevent precipitation of LDL protein during the assay, 100 μL of 0.1% (vol/vol) Triton X-100 was added before the measurement at 660 nm.

Results

LDL Receptor Expression on Human Blood Mononuclear Cells

One of the critical points in LDL receptor quantification is the question of which cell type provides the most reliable results. Therefore, in the first experiments, we compared LDL receptor expression of resting and 1 μg/mL PHA-stimulated proliferating lymphocytes with LPDS-preincubated monocytes. The fluorescence of 1 μg/mL FITC-conjugated C7A MAB bound to the cells was determined at the gated cell populations shown in Fig 1. A volume versus side-scatter contour plot of the mononuclear cells showed a distinct monocyte population to the right of the lymphocytes (Fig 1A), while proliferating lymphocytes created a population with an elongated shape with the highest density of the cells on the left, indicating different proliferation states of the lymphocytes (Fig 1B). Moreover, the larger cells that created the right population were overlapped by mononuclear phagocytes, as verified by a specific CD14 monocyte marker (data not shown). The autofluorescence of the gated cells is shown together with the FL1 of the bound C7A MAB, indicating the LDL receptor expression of monocytes (Fig 1C) and lymphocytes (Fig 1D).

The Δfluence was calculated as the difference of the mean fluorescence of FITC C7A MABs and the autofluorescence of the cells with FITC GAM. The histogram analysis showed that the autofluorescence and the fluorescence of the cell-bound MAB curves have only a small overlapping area in the monocyte as well as in the lymphocyte population, but in the case of monocytes, the Δfluence was about 60% higher. The Δfluence was determined under the same culture conditions, i.e., when monocytes and proliferating lymphocytes were labeled with Dil LDL at 4°C (Figs 1E and 1F). In contrast to proliferating lymphocytes, the fluorescence histogram of Dil LDL bound to monocytes reflecting the LDL receptor expression showed no overlap with the autofluorescence histogram. In addition, the Δmean fluorescence of the monocyte population exceeded the Δmean fluorescence of the proliferating lymphocytes by about twofold. This experiment showed that circulating human monocytes isolated from peripheral blood are able to express high amounts of LDL receptors after being cultured in LPDS for 48 hours. As shown by Knight and Soutar48 and our own data, within the time frame of our experiments significant expression of the scavenger receptors does not occur in either human serum or LPDS.
Binding Characteristics of the LDL Receptor MAB on Human Monocytes

The concentration dependency of binding of either FITC-labeled LDL receptor MABs (Figs 2A and 2B) or Dil LDL (Figs 2C and 2D) was determined on human monocytes. The receptor could be saturated at 0.1 µg/mL MAB concentration. A significant difference in LDL receptor expression could be detected between cells incubated in 10% LPDS, which causes upregulation of LDL receptors, and cells incubated in 10% human serum, in which the LDL receptor is maximally downregulated (Fig 2A). The C7A MAB was able to compete for Dil-LDL binding (Fig 2B). However, concentrations of the antibody up to 100 µg/mL medium reduced Dil-LDL binding (2 µg/mL medium of Dil LDL) in a 4°C binding experiment by about 50% (Fig 2F).
FIG 2. Line graphs showing results of binding studies on human monocytes. Panel A: Binding of the low density lipoprotein (LDL) receptor monoclonal antibody (MAB) to lipoprotein-deficient serum (LPDS)—preincubated monocytes. Monocytes were isolated from EDTA-blood by density gradient centrifugation and preincubated at 37°C in RPMI-1640 containing 10% (vol/vol) LPDS (●) or 10% (vol/vol) human serum (○) for 48 hours. Cells (4×10⁶) were incubated at 4°C with the indicated amounts of LDL receptor MAB for 30 minutes and subsequently with fluorescein isothiocyanate goat anti-mouse antibodies. Ten thousand cells were acquired in an FACSscan. The mean fluorescence values of the gated monocytes after subtraction of the autofluorescence values were plotted versus the concentration of the LDL receptor MAB used. Panel B: Competition of 3,3′-dioctadecylindocarbocyanin-iodide (Dil) LDL binding to LPDS-preincubated monocytes with a control antibody (CD14, ○) and the LDL receptor MAB (●). The cells were incubated with 10 μg/mL Dil LDL and with the indicated amounts of MAB for 1 hour at 4°C. The cells were analyzed as described for panel A. The values represent the mean of triplicate determinations; error bars signify ±SD. Panel C: Binding of Dil LDL to LPDS-preincubated monocytes. Monocytes were isolated from EDTA-blood by density gradient centrifugation and were preincubated at 37°C in RPMI-1640 containing 10% (vol/vol) LPDS (●) or 10% (vol/vol) human serum (○) for 48 hours. Cells (4×10⁶) were incubated at 4°C with the indicated amounts of Dil LDL for 1 hour. Ten thousand cells were acquired in an FACSscan. The mean fluorescence values of the gated monocytes after subtraction of the autofluorescence values were plotted versus the concentration of Dil LDL used. Panel D: The cells were incubated at 4°C for 1 hour in a constant volume and a constant mass of LDL (20 μg/mL) but with varying amounts of fluorescence-labeled lipoprotein (rhodamine isothiocyanate [RITC] LDL, ●; Dil LDL, ○), which is presented as the percentage of total LDL protein. The cells were analyzed for cell-associated fluorescence at 580 nm in an FACSscan as described for panel A. The values represent the mean of triplicate determinations; error bars signify ±SD.

2B), indicating a slightly different MAB binding site as compared with Dil LDL. A control antibody (CD14) had no effect on Dil-LDL binding.

Binding Characteristics of Dil LDL on Human Monocytes

With Dil LDL as the ligand, the receptor could be saturated at 2 μg/mL Dil LDL (Fig 2C). As with the MAB assay, the ligand assay with LPDS-preincubated cells revealed significantly higher cell-associated fluorescence compared with human serum-preincubated cells. However, the binding curve using human serum—preincubated monocytes also indicated some nonspecific Dil-LDL binding. The effect of conjugation of LDL with Dil or RITC on their ability to compete with native LDL was studied as proposed by Bohn. Monocytes were incubated in a constant volume of medium and a constant mass of total LDL (10 μg) but with varying amounts of fluorescence-labeled lipoprotein (rhodamine isothiocyanate [RITC] LDL, ●; Dil LDL, ○), which is presented as the percentage of total LDL protein. The cells were analyzed for cell-associated fluorescence at 580 nm in an FACSscan as described for panel A. The values represent the mean of triplicate determinations; error bars signify ±SD.
affinity for the LDL receptor (Fig 2D). Specific binding of Dil LDL and \( ^{125}\)I-LDL was also compared and calculated as the difference of total and nonspecific binding. In both cases the saturation was found at 2 \( \mu \text{g/mL} \) LDL concentration, and Scatchard analysis revealed similar dissociation constant \( (K_D) \) values (Dil LDL, \( 5.7 \times 10^{-9} \text{ M} \); \( ^{125}\)I-LDL, \( 8.5 \times 10^{-9} \text{ M} \)). The maximum binding capacity \( (B_{max}) \) calculated from the radioligand assay was 28 ng (Fig 3).

**Analysis of LDL Receptor Deficiencies**

The LDL receptor expression of monocytes obtained from previously diagnosed FH patients was also measured with flow cytometry using Dil LDL and FITC C7A MAB. Monocytes were isolated and cultured in LPDS for 48 hours, and their binding characteristics with Dil LDL and FITC C7A MAB were determined as described in "Methods." The heterozygous patients had about 50% reduction in the mean fluorescence of monocytes treated with either Dil LDL or FITC C7A MAB, whereas the homozygous patients expressed less than 20% of the binding activity of the control subjects (Fig 4). The LDL receptor expression was studied in the monocytes of eight FH patients with flow cytometry and with \( ^{125}\)I-LDL and \( ^{125}\)I-C7A MAB. The comparison of the results performed by the various methods is shown in Table 1. The fluorescence of the FITC C7A MAB and Dil LDL is presented as mean fluorescence, and \( ^{125}\)I-LDL and \( ^{125}\)I-C7A MAB bindings are expressed as nanograms and picograms per milligram cell protein, respectively. Finally, all the values are also calculated as percent of appropriate control subjects, which allows discrimination between heterozygous and homozygous FH patients. A cohort of 128 healthy age- and sex-matched control individuals from the clinic staff tested at six different time points with normal serum lipid parameters (total cholesterol <220 mg/dL) and negative family history of coronary heart disease was checked for LDL receptor expression with the flow-cytometric method using the C7A MAB. No correlation of mean fluorescence of the FITC C7A MAB could be observed with the age of the control individuals. The difference of mean fluorescence values ranged within \( \pm 19\% \) of the controls' mean fluorescence values. Variations in a single individual from determinations at different times were <14% (data not shown). Patients with less than 70% expression of the LDL receptor on their monocytes are considered as LDL receptor–deficient individuals: between 70% to 20% are heterozygous and less than 20% are homozygous. We
could establish the same diagnostic category in the case of each patient checked with all four methods.

**Analysis of LDL Receptor Defects**

As shown above, Dil LDL and 125I-LDL have the same binding characteristics. We could detect with fluorescence-conjugated MABs the LDL receptor deficiencies similar to 125I-LDL binding. However, our competition studies with Dil LDL and unlabeled C7A MAB indicated slightly different binding sites for the LDL receptor (Fig 2B). Therefore, it is possible to detect patients that produce and express structurally altered LDL receptors on the cell surface, which bind the antibodies but not LDL.37 Fig 5 shows the result of an experiment in which

**TABLE 1.** Comparison of Radioligand Assays With Flow-Cytometric Assays for Determination of LDL Receptor Activities on LPDS-Preincubated Monocytes of 12 Normocholesterolemic or Hypercholesterolemic Probands

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (y)</th>
<th>N/He/Ho</th>
<th>Total chol (mg/dL)</th>
<th>125I-LDL (ng bound/mg protein) (%)</th>
<th>Dil LDL Mean FL (%)</th>
<th>125I-MAB (pg bound/mg protein) (%)</th>
<th>MAB binding Mean FL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>28</td>
<td>N</td>
<td>185</td>
<td>73</td>
<td>100</td>
<td>52</td>
<td>100</td>
</tr>
<tr>
<td>J.S.</td>
<td>44</td>
<td>He</td>
<td>383</td>
<td>41</td>
<td>56</td>
<td>27</td>
<td>52</td>
</tr>
<tr>
<td>B.S.</td>
<td>42</td>
<td>He</td>
<td>345</td>
<td>47</td>
<td>65</td>
<td>25</td>
<td>48</td>
</tr>
<tr>
<td>P.S.</td>
<td>17</td>
<td>Ho</td>
<td>566</td>
<td>6</td>
<td>8</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>M.S.</td>
<td>16</td>
<td>M.o.</td>
<td>511</td>
<td>7</td>
<td>9</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>Control 2</td>
<td>35</td>
<td>N</td>
<td>195</td>
<td>68</td>
<td>100</td>
<td>47</td>
<td>100</td>
</tr>
<tr>
<td>R.F.</td>
<td>48</td>
<td>He</td>
<td>421</td>
<td>39</td>
<td>57</td>
<td>20</td>
<td>43</td>
</tr>
<tr>
<td>Control 3</td>
<td>42</td>
<td>N</td>
<td>223</td>
<td>65</td>
<td>100</td>
<td>63</td>
<td>100</td>
</tr>
<tr>
<td>G.G.</td>
<td>70</td>
<td>He</td>
<td>389</td>
<td>40</td>
<td>62</td>
<td>36</td>
<td>57</td>
</tr>
<tr>
<td>Control 4</td>
<td>26</td>
<td>N</td>
<td>175</td>
<td>77</td>
<td>100</td>
<td>58</td>
<td>100</td>
</tr>
<tr>
<td>A.G.</td>
<td>18</td>
<td>Ho</td>
<td>748</td>
<td>11</td>
<td>14</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>B.V.</td>
<td>40</td>
<td>He</td>
<td>422</td>
<td>32</td>
<td>42</td>
<td>23</td>
<td>39</td>
</tr>
</tbody>
</table>

LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; N, normocholesterolemic proband; He, familial hypercholesterolemia heterozygous patient; Ho, familial hypercholesterolemia homozygous patient; chol, cholesterol; Dil, 3,3'-dioctadecylindocarbocyanine-iodide; FL, fluorescence; MAB, monoclonal antibody.

The cells were isolated and preincubated with LPDS, and LDL receptor activity was measured by using fluorescence or radiolabeled LDL or LDL receptor MABs as described in Fig 5 and Fig 6. Bmax values were calculated from Scatchard analysis. The experiments were done in four blocks with a control for each block. The percent MAB and LDL binding activities are expressed against the appropriate control.
the patient's monocytes were able to bind the antibody normally, as indicated by a similar Δmean fluorescence in comparison to normal monocytes (Fig 5A), but the Bmax of Dil-LDL binding to the cells at 4°C was reduced by about 45% (Fig 5B). In some of the patients even higher numbers of C7A MAB binding sites were found compared with normal monocytes. With the binding of C7A MAB or Dil LDL we can detect LDL receptor deficiencies; however, this does not provide information about the defects of internalization. Therefore, we compared the 4°C binding characteristics of Dil LDL with its binding at 20°C during 60 minutes of incubation time in LPDS-preincubated monocytes, revealing bound and internalized ligand. In this assay we used monocytes from a control subject and a patient with a previously diagnosed internalization defect (Fig 6). The same level of fluorescence signal was observed at 4°C in both the control subject and the patient. However, at 20°C the patient had about 40% lower mean fluorescence values, indicating a defective uptake of fluorescence-labeled LDL particles in spite of normal LDL receptor expression. Unlabeled normal LDL can effectively compete with Dil LDL for binding. How-

**FIG 5.** Line graphs showing 4°C monoclonal antibody (MAB) binding (panel A) and 3,3'-dioctadecyldihexylcarbocyanin iodide low density lipoprotein (Dil LDL) binding (panel B) to monocytes of a hypercholesterolemic patient (total cholesterol, 326 mg/dl). Monocytes were isolated from EDTA-blood by density gradient centrifugation and preincubated at 37°C in RPMI-1640 containing 10% (vol/vol) lipoprotein-deficient serum (LPDS) (control subject, O; patient, •) or 10% (vol/vol) human serum (control subject, ©) for 48 hours. Cells (4×10^6) were incubated at 4°C with the indicated amounts of MAB and subsequently labeled with fluorescein isothiocyanate goat anti-mouse IgG (panel A) or with Dil LDL (panel B) for 1 hour. Ten thousand cells were acquired in an FACScan. The mean fluorescence values of the gated monocytes after subtraction of the autofluorescence values were plotted versus the concentration of LDL receptor MAB or Dil LDL used. The values represent the mean of triplicate determinations; error bars signify ±SD.

**FIG 6.** Line graphs showing 4°C (panel A) and 20°C (panel B) binding of 3,3'-dioctadecyldihexylcarbocyanin iodide low density lipoprotein (Dil LDL) in monocytes of a patient with an internalization defect. Monocytes were isolated from EDTA-blood by density gradient centrifugation and preincubated at 37°C in RPMI-1640 containing 10% (vol/vol) lipoprotein-deficient serum (control subject, O; patient, •) or 10% (vol/vol) human serum (control subject, ©) for 48 hours. Cells (4×10^6) were incubated at 4°C or 20°C with 10 μg/mL Dil LDL for the indicated times. Ten thousand cells were acquired in an FACScan. The mean fluorescence values of the gated monocytes after subtraction of the autofluorescence values were plotted versus the concentration of Dil LDL used. The values represent the mean of triplicate determinations; error bars signify ±SD.
Among Hypercholesterolemic Patients

Lipoprotein (apo) B 3500-defective low density lipoprotein (LDL) for 4°C 3,3’-dioctadecylindocarbocyanin-iodide (DiI) LDL binding to the U937 monocytic cell line. U937 monocytes were cultured at 37°C in RPMI-1640 containing 10% (vol/vol) lipoprotein-deficient serum for 48 hours. The cells were then incubated with 10 μg/mL DiI LDL and with the indicated amounts of unlabeled native LDL (●) or apoB 3500-defective LDL (●) for 1 hour at 4°C. Ten thousand cells were acquired in an FACSscan. The mean fluorescence values of the gated monocytes after subtraction of the auto-fluorescence values were plotted versus the concentration of unlabeled lipoproteins used. The values represent the mean of triplicate determinations; error bars signify ±SD.

Differential Diagnosis of LDL Receptor Defects Among Hypercholesterolemic Patients

Using this elaborated methodology, we analyzed a preselected cohort of 384 hypercholesterolemic patients and their relatives for LDL receptor defects. We determined total cholesterol, triglyceride, LDL-C, HDL-C, and Lp[a] levels, apoB 3500 mutants, and apoE isoforms. The LDL receptor expression was studied as a quick screening method with FITC-conjugated C7A MABs on human cultured monocytes obtained from the patients. Based on their total cholesterol levels, patients were categorized into three groups with different levels of hypercholesterolemia: group I, mild (<250 mg/dL); group II, moderate (250 to 300 mg/dL); and group III, severe (>300 mg/dL), according to the guidelines for management of hyperlipidemia of the European Atherosclerosis Society69 (Table 2). The distribution of unrelated hypercholesterolemic patients and relatives of unrelated FH is also shown for each group. Patients without LDL receptor deficiency measured by the binding of FITC C7A MABs were further investigated for LDL receptor defects. In these cases binding defects were analyzed with DiI LDL at 4°C, and internalization defects were analyzed with 20°C uptake. In group III, 71.8% of the patients exhibited LDL receptor deficiencies, and the frequency was the same among unrelated individuals and relatives. Five patients in this group exhibited LDL receptor expression below 20% of control subjects, indicating homozygous FH. The average concentration of Lp[a] was also the highest in this group. In group II the percentage of LDL receptor-deficient subjects was 51.3%, although its occurrence was 75% among the relatives. In group I, 24.7% of the individuals were found to have decreased LDL receptor expression, but in the case of relatives its frequency was only 8%. In groups I and II, no FH homozygous patients were detected. Patients with FDB were found only in groups II and III. The studies with apoE isoforms showed that apoE3/3 is the most frequent phenotype in all groups, followed by the apoE4/3 and apoE3/4 phenotypes. Less than 4% of all patients analyzed belonged to the apoE2/2, E2/4, or E4/4 phenotype. The apoE polymorphism study showed no significant correlation with serum cholesterol levels and LDL receptor deficiencies for the apoE2/3, E2/4, or E3/3 phenotypes. Patients homozygous for the apoE2 allele (n=2) had cholesterol levels above 300 mg/dL. The prevalence of the apoE3/4 phenotype was highest in group II, whereas patients homozygous for the apoE4 allele were found in either group I or group III.

Table 2. Lipid Parameters Among 384 Normocholesterolemic and Hypercholesterolemic Subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>&lt;250</td>
<td>250-300</td>
<td>&gt;300</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>135±25</td>
<td>203±24</td>
<td>307±79</td>
</tr>
<tr>
<td>Patient distribution (of total; n=384)</td>
<td>32.0 (123)</td>
<td>22.1 (85)</td>
<td>45.9 (176)</td>
</tr>
<tr>
<td>Unrelated (% of groups I-III)</td>
<td>78 (96)</td>
<td>67 (57)</td>
<td>73 (128)</td>
</tr>
<tr>
<td>Related (% of groups I-III)</td>
<td>22 (27)</td>
<td>33 (28)</td>
<td>27 (48)</td>
</tr>
<tr>
<td>Phenotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2/2 (%)</td>
<td>0.0</td>
<td>0.0</td>
<td>1.3 (2)</td>
</tr>
<tr>
<td>E2/3 (%)</td>
<td>3.2 (4)</td>
<td>10.0 (8)</td>
<td>5.3 (9)</td>
</tr>
<tr>
<td>E2/4 (%)</td>
<td>1.6 (2)</td>
<td>3.3 (3)</td>
<td>2.6 (4)</td>
</tr>
<tr>
<td>E3/3 (%)</td>
<td>62.9 (77)</td>
<td>46.7 (40)</td>
<td>56.6 (99)</td>
</tr>
<tr>
<td>E4/3 (%)</td>
<td>21.0 (26)</td>
<td>40.0 (34)</td>
<td>31.6 (57)</td>
</tr>
<tr>
<td>E4/4 (%)</td>
<td>11.3 (14)</td>
<td>0.0</td>
<td>2.6 (5)</td>
</tr>
<tr>
<td>Lp[a] &gt;25 mg/dL (%)</td>
<td>22.6 (28)</td>
<td>34.0 (29)</td>
<td>36.1 (64)</td>
</tr>
<tr>
<td>FDB (%)</td>
<td>0.0</td>
<td>4.7 (4)</td>
<td>1.7 (3)</td>
</tr>
<tr>
<td>LDL receptor &lt;70% of control (of total; n=384)</td>
<td>24.7 (30)</td>
<td>51.3 (44)</td>
<td>71.8 (126)</td>
</tr>
<tr>
<td>Unrelated (% in groups I-III)</td>
<td>29 (28)</td>
<td>40 (23)</td>
<td>71 (91)</td>
</tr>
<tr>
<td>Related (% in groups I-III)</td>
<td>8 (2)</td>
<td>75 (21)</td>
<td>73 (25)</td>
</tr>
</tbody>
</table>

LDL-C, low density lipoprotein cholesterol; Lp[a], lipoprotein[a]; FDB, familial defective apolipoprotein B-100; LDL receptor expression was determined by using the LDL receptor monoclonal antibody and fluorescein isothiocyanatelabeled goat anti-mouse antibodies as described in Fig 4. The values represent the percentage of LDL receptor deficiencies found in either cohort and the numbers in parentheses show the number of patients in each group. An LDL receptor deficiency was assumed if the mean fluorescence values of the monocytes were below 70% of the control subjects’ values. The border of reduced LDL receptor expression derives from measurements performed among healthy control individuals as described in “Methods.” The apoE polymorphism, apoB-100 mutation, and Lp[a] levels were determined as described in “Methods.” The values for Lp[a] represent the percentage of patients in either cohort having Lp[a] levels higher than 25 mg/dL.
Discussion

Lymphocytes were the first blood cells shown to have LDL receptor activity by an ex vivo assay. In FH, it is apparent that a reduction in LDL receptor numbers on the surface of circulating leukocytes is not itself a cause of high serum cholesterol levels but rather reflects the reduced LDL receptor activity in other cells, especially in the liver. There is, however, now no doubt about the clinical usefulness of measuring the expression of LDL receptor activities on circulating leukocytes as a marker for LDL receptor defects.

The determination of various receptors on the surface of blood cells seems to be a well-established widespread method in the clinical diagnosis of several endocrinologic disorders. However, the value of a particular receptor assay on circulating blood cells must be proven for each receptor type and each disease. The LDL receptor determination on various cell types has shown already that the features of the classic LDL receptor pathways are common to all cell types of the human organism. However, the level of LDL receptors is influenced by the presence of regulators, such as the concentration of LDL in the surrounding medium, and also by the metabolic activity and proliferation of the cells. Therefore, experiments were performed on human monocytes and lymphocytes cultured for 48 hours in LPDS, and the LDL receptor expression was determined in flow-cytometric assays with FITC-conjugated C7A MABs or Dil LDL and compared with 125I-LDL binding. The same binding characteristics and affinities could be detected, as has already been described for the classic LDL receptor. The only marked difference between monocytes and lymphocytes was a higher LDL receptor expression on monocytes. The difference between the two cell types in LDL receptor expression can be explained by the fact that lymphocytes preferentially synthesize endogenous cholesterol instead of relying on exogenous uptake by the LDL receptor, which is just the opposite in monocytes/macrophages and fibroblasts. The highest rate of LDL receptor expression in lymphocytes could be induced only by proliferation. However, proliferating lymphocytes created a lower mean fluorescence compared with monocytes, and no homogenous LDL receptor active population could be gated in the volume versus side-scatter contour plots. Within the lymphocyte population, CD4 phenotype cells have a higher receptor activity than CD8 cells. Furthermore, in CD8 cells the low-affinity immunoregulatory receptor may be responsible for the uptake of apoB/E-containing lipoproteins, in addition to the native LDL receptor. Some functional LDL receptor assays have also been developed for lymphocytes. Lymphocytes with normal LDL receptor activity proliferate in very low concentrations of LDL, whereas those of FH homozygotes require high and FH heterozygotes require intermediate concentrations. However, there are substantial additional reasons why the use of lymphocytes is unsatisfactory. Lymphocyte mitogenesis decreases during aging. On the other hand, the LDL receptor activity of elderly people increases in lymphocytes. Thus, taking into account our experience and information in the literature, monocytes are preferred for the detection of LDL receptor deficiencies by flow cytometry.

The C7A MAB and Dil LDL turned out to be suitable probes for the determination of LDL receptor activities. The MAB reduces Dil-LDL binding by about 45%, which is in agreement with other studies. However, a cross-reactivity with the LDL receptor-related protein, which seems to be expressed only on liver cells, cannot be ruled out. In the competition experiment, Dil LDL revealed similar binding characteristics to native LDL, whereas RITC LDL showed an altered binding affinity to the receptor. Flow-cytometric LDL receptor measurement with FITC C7A MAB on cultured human monocytes gave similar results to other binding assays (eg, Dil LDL, 125I-MAB, and 125I-LDL) for the detection of LDL receptor deficiencies in the eight FH patients studied. A clear discrimination between the heterozygous and homozygous forms of FH can be obtained using the flow-cytometric assays (Fig 4). Moreover, by the combined use of fluorescence-labeled antibodies and Dil LDL in a 4°C binding assay (Fig 5), LDL receptor deficiencies can be discriminated clearly from LDL receptor defects, as Beisiegel et al demonstrate by using radiolabeled probes. Internalization defects can be detected by comparing 4°C with 20°C Dil-LDL binding characteristics (Fig 6). The competition assay shown in Fig 7 indicates that it might also be possible to detect ligand defects, such as FDB-caused hypercholesterolemia, if the patient’s own LDL is used for the competition of Dil-LDL binding to LDL receptor–positive normal monocytes.

Finally, we analyzed a preselected cohort of 384 hypercholesterolemic patients, including the relatives of FH patients, based on the flow-cytometric measurement of LDL receptor activity of monocytes cultured for 48 hours in LPDS. On the basis of their serum cholesterol levels, patients were divided into three groups. In the first group (serum cholesterol <250 mg/dL), 24.7% of the patients were found, surprisingly, to have LDL receptor activities below 70% of control subjects’ levels, ie, to be receptor deficient. A detailed analysis revealed two reasons for these anomalous cases. First, a few patients (n=9) had been treated by LDL apheresis or hydroxymethylglutaryl coenzyme A reductase inhibitors at the time of our investigations. Thus, the serum cholesterol levels of these patients reflected the effectiveness of the lipid lowering, while the flow-cytometric assay still revealed the underlying LDL receptor defect. Second, some patients were children aged less than 10 years (n=18), in whom hypercholesterolemia should be diagnosed when total cholesterol is above 200 mg/dL rather than 250 mg/dL. It can be also mentioned that during the family studies the occurrence of normocholesterolemia with reduced LDL receptor activity was found in three untreated relatives belonging to the same family (a father and two sons), which is similar to the case described by Vega et al. This might indicate the presence of the cholesterol-lowering gene described by Hobbs et al, but this needs further analysis. All of the patients in group III with serum cholesterol >300 mg/dL had the clinical diagnosis of FH. However, 28.2% of the patients had reasons for hypercholesterolemia other than LDL receptor deficiencies, and the distribution among unrelated individuals and relatives was similar. In group II all of the relatives and 85% of the unrelated individuals had the clinical diagnosis of FH. Only 75% of the related and 40% of the unrelated
hypercholesterolemic subjects were found to have LDL receptor deficiency in the flow-cytometric assay. The major problem with the clinical diagnosis of FH is that the clinical criteria cannot be well defined. If xanthomas are required to make the diagnosis of FH, one will definitely underestimate the true prevalence of the disease. On the other hand, if one relies only on the presence of hypercholesterolemia in a kindred, the prevalence of FH will probably be overestimated. This appears to be the case in our patient population. However, the flow-cytometric LDL receptor assay has been designed to overcome this problem. It appears to be particularly useful in patients with moderate hypercholesterolemia (250 to 300 mg/dL), who are difficult to classify using only clinical criteria.

In two patients belonging to the same family the flow-cytometric assay revealed normal antibody binding characteristics but reduced Dil-LDL uptake at 20°C, thus showing an internalization defect. A mutant apoB 3500 in three patients from group III and four patients from group II was verified by allele-specific oligonucleotide mutants and, additionally, by the competition assay shown in Fig 7. The frequency of the apoB 3500 mutation among our hypercholesterolemic patients is about 3%, similar to that observed among other western European populations. On the basis of their medical history, four patients had secondary forms of hypercholesterolemia due to endocrinologic disorders. Twenty-two patients seemed to have nongenetic, dietary-induced hypercholesterolemia. It might be possible that among these 22 patients the pseudohomozygous type II hyperlipoproteinemia, in which hypercholesterolemia with normal LDL receptor expression occurs, can be found.

In the investigated cohort of hypercholesterolemic patients, the occurrence of certain apoE phenotypes did not directly correlate with serum cholesterol levels and LDL receptor defects. Patients with the apoE2/2 phenotype (n=2) had cholesterol values above 300 mg/dL, which for this phenotype is unusually high, but they also exhibited an LDL receptor expression below 70% of control subjects, indicating a combined genetic disorder. The homozygous apoE4 isoform with the highest affinity to the LDL receptor among the apoE isoforms occurred in group I as well as in group III, whereas the Lp[a] concentration was highest in group III. In some patients these findings could be responsible for the hypercholesterolemia, indicating that the effects of the apoE phenotype and Lp[a] on hypercholesterolemia are independent of the LDL receptor status. Lp[a] levels are not associated with defects in the LDL receptor gene, but they are associated with the mutation of apoB 3500.

In summary, flow-cytometric measurement with C7A MAB in human monocytes cultured for 48 hours in LPDS seems to be a rapid, simple method in the screening and clinical diagnosis of hypercholesterolemia and in the detection of genetic causes for reduced LDL receptor expression. Such a method would contribute to increasing the effectiveness of coronary artery disease prevention. The introduction of LDL receptor and apoE isofrom analysis and Lp[a] detection at the routine diagnostic level in lipid centers may reveal frequent causes of hypercholesterolemia among a given population.


Fluorescence flow cytometry of human leukocytes in the detection of LDL receptor defects in the differential diagnosis of hypercholesterolemia.

G Schmitz, T Brüning, E Kovacs and S Barlage

*Arterioscler Thromb Vasc Biol.* 1993;13:1053-1065
doi: 10.1161/01.ATV.13.7.1053

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 1993 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/13/7/1053

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at:
http://atvb.ahajournals.org/subscriptions/