Monocytes From Patients With Combined Hypercholesterolemia-Hypertriglyceridemia and Isolated Hypercholesterolemia Show an Increased Adhesion to Endothelial Cells In Vitro: II. Influence of Intrinsic and Extrinsic Factors on Monocyte Binding

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One of the primary risk factors for atherosclerosis is hypercholesterolemia. Patients with isolated hypercholesterolemia or combined hypercholesterolemia-hypertriglyceridemia are at risk to develop premature atherosclerosis. Diet-induced hypercholesterolemia in animals leads to an increased adhesion of monocytes to and transmigration through the intact endothelium of the vessel wall. In the present study, we investigated in vitro binding of freshly isolated monocytes from patients and healthy controls to a monolayer of endothelial cells obtained from human umbilical vein. All four diagnosed patient groups with isolated or combined hypercholesterolemia showed a significant increase in monocyte binding as compared with the control group (familial hypercholesterolemia [FH], +41%; polygenic hypercholesterolemia [PH] +35%; familial combined hypercholesterolemia [FCH], +47%; nonfamilial combined hypercholesterolemia-hypertriglyceridemia [CHH], +67%). In a longitudinal study it was observed that diet or medication induced a decrease in cholesterol and triglycerides; however, these therapeutic conditions did not diminish in vitro monocyte binding in the patient groups. There was no correlation between monocyte binding and plasma cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, or lipoprotein(a) within hyperlipidemic patient groups. The presence of heart and vessel disease in hyperlipidemic patients was not associated with a change in monocyte binding. The adhesion to endothelial cells of monocytes from smoking patients with combined hypercholesterolemia (27%) was significantly higher (+23%) than that of monocytes from nonsmoking patients. Cytofluorimetric analysis of monocytes from FCH and CHH patients for specific monocyte differentiation markers and integrins did not show differences as compared with monocytes from healthy controls.

HYPERCHOLESTEROLEMIA is one of the main risk factors for development of premature atherosclerosis and coronary heart disease. Cholesterol levels are directly related to this increased risk.[1,2] For patients with combined hypercholesterolemia-hypertriglyceridemia, the increased risk for premature atherosclerosis is mainly attributed to elevated serum cholesterol levels,[3,4] but elevated serum total triglycerides may also be important.[5]

Diet-induced hypercholesterolemia in animals produced an increased adhesion of monocytes to the endothelium of the vessel wall. After adhesion, monocytes migrate to the subendothelium, differentiate into macrophages, take up lipids, and become foam cells.[6-10] So increased monocyte adhesion to endothelium is thought to be the first event in the development of fatty streaks.

In an earlier preliminary study in a small group of hyperlipidemic patients, we found a significant increase in monocyte adhesion as compared with monocytes from healthy subjects.[11] In this extended study, we investigated in vitro monocyte-binding characteristics of larger groups of patients with isolated hypercholesterolemia or combined hypercholesterolemia-hypertriglyceridemia. Monocytes were also analyzed for specific monocyte differentiation markers and integrins by flow cytometry. Furthermore, in a longitudinal study the influence of a cholesterol-lowering diet or medication on monocyte binding was evaluated. Serum factors such as total cholesterol, total triglycerides, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and lipoprotein (a) were correlated with monocyte binding. Also, features such as the presence of heart and vessel disease, gender, or smoking behavior were assessed.

SUBJECTS AND METHODS

Patients

All patients were referred to our lipid clinic because of hyperlipidemia (October 1991 through March 1993). At each patient's initial visit, a complete physical examination and detailed history were obtained, as well as family history. Weight and height were measured, the Quetelet index was calculated, and patients were seen by a dietician. Blood was drawn after a 12-hour fast for measurement of cholesterol, triglyceride, and HDL cholesterol levels. To rule out common secondary causes of hyperlipidemia, liver, renal, and thyroid function, and glucose levels were measured. Patients with diabetes mellitus, hypothyroidism, or any other cause of secondary hyperlipidemia were excluded from the study.

Familial hypercholesterolemia (FH)[12] was identified by the presence of an isolated increased plasma cholesterol level, family history, and tendonous xanthomata in the patient or his first-degree pedigree. Polygenic hypercholesterolemia (PH)[12] indicated an isolated increased serum cholesterol level (> 6.5 mmol/L) without a family history of either dyslipidemia or premature coronary heart disease. Familial combined hyperlipidemia (FCH)[12] indicated increased serum cholesterol (> 6.5 mmol/L) and serum triglyceride (> 2.5 mmol/L) levels and a family history of both dyslipidemia and premature heart disease. Nonfamilial combined hypercholesterolemia-hypertriglyceridemia (CHH)[11] indicated increased serum cholesterol and serum triglyceride levels without a

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family history of either dyslipidemia or premature coronary heart disease.

Blood for the present study was obtained from fasted patients before lipid-lowering regimens were started. For the longitudinal study on the effect of a cholesterol-lowering diet with or without medication, blood was also taken after several months of treatment (isolated hypercholesterolemia, hepatic hydroxymethyl glutaryl coenzyme A reductase inhibitor; combined hypercholesterolemia, hepatic hydroxymethyl glutaryl coenzyme A reductase inhibitor [n = 3], acipimox [n = 2], and gemfibrozil [n = 2]). As a control, the monocyte binding of fasting healthy controls was compared before and after an interval of 6 months.

Isolation of Human Monocytes

Monocytes were isolated from 10 mL anticoagulated (EDTA) whole blood collected from fasting normal donors and patients. First, mononuclear cells were obtained by gradient centrifugation using Ficoll-Paque (Pharmacia Biotech Europe, Brussels, Belgium). The cells, which were collected from the interface, were washed twice by centrifugation (10 minutes, 100 × *g*), and monocytes were enriched by centrifugation on a Percoll gradient ($\rho = 1.063$; Pharmacia).[13] Purity of the monocyte fraction was determined by staining a sample of the cells with fluorescein isothiocyanate (FITC)-conjugated CD14 monoclonal antibody (My4; Coulter, Hialeah, FL).

Isolation of Endothelial Cells

Venous endothelial cells (HUVEC) were isolated from human umbilical vein with 0.1% collagenase using the method reported by Jaffe et al.[14] Cells were plated in culture flasks precoated with fibronectin (10 $\mu g/cm^2$) and grown to confluence in culture medium.[15]

Adherence Assay

Suspensions of monocytes were incubated with the fluorescent dye 5- and 6-carboxy-fluorescein diacetate succimidylester 30 μmol/L for 10 minutes at 37°C (Molecular Probes, Eugene, OR).[16] Confluent endothelial layers (passages 2 or 3, confluent for at least 4 days on 15-mm [2-cm^2] Thermanox coverslips [Lux 5414; Nunc, Naperville, IL]) were incubated in 1 mL culture medium containing 4×10^5 monocytes for 60 minutes at 37°C under static conditions. Nonadherent cells were washed off by dipping the cover slip twice in medium M-199 (Flow Laboratories, Irvine, Scotland) and once in phosphate-buffered saline.[11] After fixation (3% paraformaldehyde for 20 minutes), the number of bound monocytes per millimeter squared was counted using a fluorescence microscope. Adherent monocytes were expressed as percent of total monocytes added.

Total Cholesterol and Triglyceride Determinations

Plasma concentrations of triglyceride and cholesterol were determined by enzymatic methods (Boehringer testkit combination, Boehringer, Mannheim, Germany). Human HDL cholesterol level was measured after precipitation of very-low-density lipoprotein and LDL with dextran sulfate–magnesium chloride by an enzymatic method (Boehringer testkit combination). Lipoprotein(a) level was measured using an immunoradiometric assay (Pharmacia).

Flow Cytophotometry of Monocytes

Mononuclear cells obtained by Ficoll-Paque centrifugation were stained with monoclonal antibodies according to the manufacturer's instructions. The following conjugated monoclonal antibodies were used: anti-CD11a–FITC, anti-CD18–FITC (Immunotech, Marseille, France), anti-CD14 (anti-My4)–FITC, anti-CD14 (anti-My4)–phycoerythrin (PE) (Coulter), anti-CD11b–PE, anti-CD11c–PE, and anti-CD16–FITC (Becton Dickinson, San Jose, CA). Cells were analyzed on a FACSCAN flow cytometer (Becton Dickinson).

Statistics

Differences between groups were analyzed using Student's *t* test. Correlation analysis was performed using Spearman's rank method. The influence of diet or medication on monocyte binding was tested using Student's paired *t* test.[17]

RESULTS

Monocyte Binding and Plasma Components

In an earlier study,[11] the adherence of monocytes from patients with hyperlipidemia to cultured endothelial cells was studied in a small group of patients. In this investigation with a greater number of patients, there was again a significantly increased binding of monocytes to endothelium, but now in all patient groups, as compared with that of monocytes from healthy controls (Table 1). The group with the greatest degree of monocyte binding (CHH) differed significantly from all other patient groups (*t* test, $P < .05$). There was no correlation between individual monocyte binding and levels of plasma components [total cholesterol, HDL cholesterol, LDL cholesterol, total triglycerides, or lipoprotein(a)] within different patient groups.

Monocyte Binding: Diet or Medication

In a smaller patient group, we investigated whether diet or medication had any influence on monocyte binding and

Table 1. Monocyte Adhesion to Endothelial Cells (mean ± SEM)

Monocyte Source	Adherent Monocytes (% binding)*	Plasma Cholesterol (mmol/L)	Plasma Triglycerides (mmol/L)	Serum HDL Cholesterol (mmol/L)	Plasma Lipoprotein (a) (U/L)
Healthy controls (n = 18)	15.4 ± 0.82	5.2 ± 0.19	0.9 ± 0.05	1.47 ± 0.070	ND
FH (n = 28)	21.6 ± 0.99†	9.3 ± 0.36†	1.7 ± 0.16†	1.18 ± 0.090†	404 ± 85.2
PH (n = 10)	20.7 ± 1.65†	7.7 ± 0.55†	1.7 ± 0.16†	1.17 ± 0.054†	389 ± 119.7
FCH (n = 17)	22.2 ± 1.34†	7.9 ± 0.36†	5.5 ± 1.17†	0.88 ± 0.042†	416 ± 138.3
CHH (n = 17)	25.4 ± 1.44†	8.5 ± 0.48†	7.3 ± 2.64†	0.87 ± 0.061†	252 ± 91.9

Abbreviation: ND, not determined.

*Percentage of monocytes that bound to a monolayer of endothelial cells in 1 hour at 37°C.

†Significantly different as compared with healthy donors (Student's *t* test, $P < .05$).

Table 2. Influence of Diet and Medication on Monocyte Binding and Plasma Lipid Levels (mean ± SEM)

Monocyte Source	% Adherent Monocytes		Cholesterol (mmol/L)		Triglycerides (mmol/L)		HDL Cholesterol (mmol/L)	
	Before	After	Before	After	Before	After	Before	After
Diet								
Isolated HC (FH + PH, n = 8)	18.7 ± 1.09	24.3 ± 6.30	7.6 ± 0.40	6.4 ± 1.11*	1.5 ± 0.22	1.7 ± 0.19	1.2 ± 0.10	1.1 ± 0.06
HC/HT (FCH + CHH, n = 9)	21.9 ± 1.7	32.3 ± 9.0	7.7 ± 0.6	7.3 ± 0.8	6.6 ± 2.17	6.7 ± 3.07	0.8 ± 0.06	0.8 ± 0.07
Medication								
Isolated HC (FH, n = 6)	22.7 ± 1.5	31.5 ± 8.5	9.0 ± 0.46	6.3 ± 0.44*	1.7 ± 0.33	1.3 ± 0.28*	1.2 ± 0.11	1.6 ± 0.37
HC/HT (FCH + CHH, n = 7)	22.9 ± 2.17	20.7 ± 5.05	7.8 ± 0.69	7.2 ± 0.94	4.4 ± 0.67	5.5 ± 2.89	0.79 ± 0.06	0.88 ± 0.07

Abbreviations: HC, hypercholesterolemia; HT, hypertriglyceridemia.
*Significantly different from before (Student's paired t test, $P < .05$).

plasma lipid levels (Table 2). Statistically (paired t test), diet did not influence monocyte binding in both patient groups (FH + PH and FCH + CHH). There was a significant decrease in cholesterol in patients with isolated hypercholesterolemia. Diet did not influence lipoprotein levels in patients with combined hyperlipidemia. Medication did not influence monocyte binding in both patient groups (FH and FCH + CHH). Medication had the expected effect in FH patients of decreasing cholesterol and triglyceride levels, but did not influence cholesterol and triglyceride levels in patients with combined hypercholesterolemia (both cases $P > .50$).

To investigate reproducibility of the measurement of monocyte binding, we examined the binding of monocytes from blood samples taken from each of nine fasted healthy controls before and after a 6-month interval. Monocytes did not differ in percentage (mean ± SEM) of binding to endothelium (first sampling, 15.4% ± 1.28%; second sampling, 15.9% ± 0.96%; paired t test, $P = .82$, n = 9). Monocyte binding in controls never surpassed 20%.

Monocyte Binding: Smoking, Age, Gender, or Presence of Disease

In the group of patients with combined hypercholesterolemia (FCH + CHH), there was a significant increase in monocyte binding in smokers (smokers, 27%, n = 12; nonsmokers, 22%, n = 17; $P = .04$). No differences were present in other patient groups and controls. There was no correlation between age and monocyte binding in controls and different patient groups, and there were no differences in monocyte binding between men and women. The presence or absence of heart and vessel disease in different patient groups did not influence monocyte binding.

Flow Cytophotometry of Monocytes

Four-parameter flow cytophotometry was performed on the Ficoll fraction of peripheral blood from healthy controls (range of monocyte binding, 11.5% to 21.5%; n = 12), FCH patients (range of monocyte binding, 17.7% to 39.5%; n = 10), and CHH patients (range of monocyte binding, 19.9% to 30.5%; n = 4). The surface antigens investigated were the integrin molecules CD18, CD11a, CD11b, and CD11c, and the differentiation antigens, CD14 and CD16. Figure 1 shows the analysis of monocytes from a patient with a high degree of in vitro monocyte binding (39.5%) as compared with monocytes from a healthy control. There were no differences in surface-antigen expression of monocytes from the patient and the healthy control.

Furthermore, in control, FCH, and CHH groups, we determined the percentage of monocytes with a simulta-

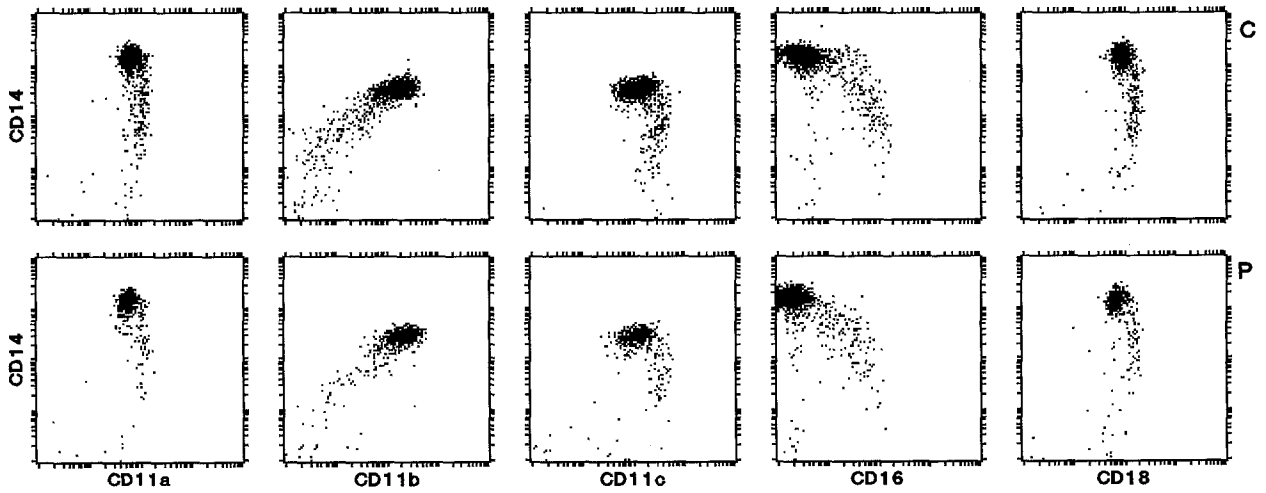

Fig 1. Flow cytophotometric analysis of monocytes. Each dot plot contains 1,000 events. Upper row, healthy donor; lower row, FCH patient. Monocytes were gated on the basis of forward and perpendicular light scatter properties. X-axis: CD11a, CD11b, CD11c, CD18, CD16, CD18. Y-axis: CD14.

neous high level of expression of CD16 and low level of expression of CD14, which seems to be an important marker population for the assessment of cardiovascular risk and hypercholesterolemia.[18] There were no significant differences ($P > .20$) between the mean (±SEM) percentages of this monocyte subpopulation in control and patient groups (control, 11.3% ± 1.44%, n = 12; FCH, 12.7% ± 1.09%, n = 10; CHH, 13.0% ± 1.25%, n = 4). There were also no other differences in the flow pattern of monocyte populations of controls and FCH and CHH patients.

DISCUSSION

In patients with isolated or combined hypercholesterolemia, blood cells and endothelial cells are in contact with high LDL cholesterol levels and sometimes with additional high very–low-density lipoprotein triglyceride levels. It seems possible that this long-term exposure plays an activating role in the process of atherosclerosis. Erythrocyte membranes from patients with severe hypercholesterolemia were significantly less fluid than respective membranes from normocholesterolemic controls,[19] although other studies suggest that hypercholesterolemia does not induce marked changes in lipid fluidity of erythrocytes and platelets.[20] Furthermore, hypercholesterolemia induces functionally abnormal monocytes with respect to eicosanoid metabolism, O_2 generation, and adhesion to glass surfaces,[21] whereas monocyte lipid levels are similar in patients and controls.[22]

In a recent study, it was shown that in vitro incubation with LDL increases monocyte binding in which the LDL receptor is involved.[23] It is possible that the increased adhesiveness of monocytes from patients with hypercholesterolemia reported in this study is also due to high LDL cholesterol levels. However, there was no correlation between LDL cholesterol levels and monocyte binding within different patient groups. Diet or medication decreases plasma triglyceride and/or cholesterol levels only moderately. It is possible that control lipoprotein levels must be reached before a significant decrease in monocyte binding can take place. This could explain the lack of correlation between binding and lipoprotein levels in patient groups.

Recently, it has been suggested that the heterogeneity of monocyte populations as visualized by flow cytophotometry of CD14 and CD16 expression might be linked to the pathogenesis of coronary artery disease or the presence of familial hypercholesterolemia (homozygous).[18] In our flow cytophotometric studies of monocytes from FCH patients, no changes were observed in CD14 and CD16 expression with respect to control monocytes (Fig 1). In the analysis of CD14 and CD16 expression of monocytes from a group of patients with combined hypercholesterolemia (FCH + CHH), the presence or absence of atherosclerotic lesions did not influence antigen expression (results not shown, $P = .74$).

A factor that could not be taken into account in this study was the long-term exposure of endothelial cells to high levels of cholesterol and lipids. It is possible that with hypercholesterolemia, endothelial cells are activated in exposing surface-binding molecules and thereby promote the subsequent binding and migration of monocytes.

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