Expression of the monocyte chemoattractant protein-1 receptor CCR2 is increased in hypercholesterolemia: differential effects of plasma lipoproteins on monocyte function

Ki Hoon Han,* Ki Ok Han,† Simone R. Green,* and Oswald Quehenberger[1,*]

Department of Medicine,* University of California, San Diego, La Jolla, CA 92093-0682, and Department of Medicine,† Samsung Cheil Women's Healthcare Center and Hospital, Sungkyunkwan University College of Medicine, Seoul, Korea

Abstract **Monocytes are recruited from the circulation into the subendothelial space where they differentiate into mature macrophages and internalize modified lipoproteins to become lipid-laden foam cells. The accumulation of monocytes is mediated by the interaction of locally produced chemoattractant protein-1 (MCP-1) with its receptor CCR2. The objective of the present study is to demonstrate the differential effects of plasma lipoproteins on monocyte CCR2 expression. The CCR2 expression was increased about 2.4-fold in monocytes isolated from hypercholesterolemic patients, compared to monocytes from normal controls. There was a significant correlation between CCR2 expression and plasma low density lipoprotein (LDL). Elevated levels of high density lipoprotein (HDL) blunted and even reverted the effects of LDL on CCR2 expression, both in vivo and in vitro. The causal relationship between plasma lipoproteins and CCR2 expression was further confirmed by modulating the lipoprotein profile. Estrogen supplement therapy decreased plasma LDL cholesterol, increased plasma HDL cholesterol, and reduced CCR2 expression in hypercholesterolemic postmenopausal women, but had no effect on the plasma lipid profile or CCR2 expression in normocholesterolemic subjects. The physiological significance of altered CCR2 expression was tested by chemotaxis assay, and our results demonstrated that treatment of THP-1 monocytes with LDL induced CCR2 expression and substantially enhanced the chemotaxis elicited by MCP-1. Our findings suggest that plasma lipoproteins differentially control monocyte function and that monocytes from hypercholesterolemic subjects are hyperresponsive to chemotactic stimuli. This may increase their accumulation in the vessel wall and accelerate the pathogenic events of atherogenesis.**—Han, K. H., K. O. Han, S. R. Green, and O. Quehenberger. **Expression of the monocyte chemoattractant protein-1 receptor CCR2 is increased in hypercholesterolemia: differential effects of plasma lipoproteins on monocyte function.** ***J. Lipid Res.*** **1999.** 40: **1053–1063.**

Supplementary key words CCR2 • MCP-1 • chemokine • lipoprotein • monocyte • atherosclerosis

Excessive recruitment of monocytes to the vessel wall is one of the earliest detectable events in human and experimental atherosclerosis. Their cytokine and growth factor-induced maturation to macrophages and their transformation to lipid-laden foam cells constitute important stages in the initiation and progression of plaques of atherosclerosis (1–3). Substantial evidence supports a role for monocyte chemoattractant protein 1 (MCP-1) in the recruitment of monocytes to the subendothelial space of arteries (4). MCP-1 belongs to the family of the C-C or β chemokines and is characterized by its ability to evoke chemotaxis mainly in monocytes (5, 6), T-lymphocytes (7), and natural killer cells (8). It is expressed by a variety of cells such as fibroblasts, vascular endothelial cells, vascular smooth muscle cells, and monocytes/macrophages in response to pro-inflammatory stimuli (9, 10). Based on these properties, MCP-1 has been suggested to play an important role in attracting specific leukocytes to sites of inflammation (11).

Despite the beneficial role of leukocyte during inflammation, a poorly regulated inflammatory response can result in tissue damage and cause harm. A sustained expression of MCP-1 was reported in a number of diseases that are characterized by monocyte infiltration, such as rheumatoid arthritis (12), and the secretion of MCP-1 by cells of the vessel wall may be central to the pathogenesis of atherosclerosis (13). It is triggered by various stimuli including low density lipoprotein (LDL) that has undergone minimal oxidative modification (14, 15), cytokines and growth factors (10, 16, 17) and exposure of the endothelium to fluid mechanical forces (18).

Abbreviations: MCP-1, monocyte chemoattractant protein 1; CCR2, receptor for MCP-1; LDL, low density lipoprotein; HDL, high density lipoprotein; LDL-C, LDL cholesterol; HDL-C, HDL cholesterol.

[1] To whom correspondence should be addressed.

Chemokines induce cell migration by binding to distinct seven-transmembrane-spanning surface receptors that signal through heterotrimeric G-protein complexes. The receptor for MCP-1 (CCR2) was identified and shown to exist in two highly homologous isoforms that differ only in the intracellular carboxyl tails (19). CCR2B appears to be the predominant form found on human monocytes, but the exact cellular localization and function of CCR2A remain uncertain (20).

The CCR2 expression on leukocytes is highly regulated and can vary depending on the microenvironment. Recent studies in our laboratory have demonstrated that pro-inflammatory cytokines such as IFN-γ, IL-1, or TNF rapidly decreased CCR2 gene expression, which may help retain monocytes at sites of inflammation and prevent their reverse transmigration (21). Macrophage maturation factors had similar effects on CCR2 expression, and differentiated macrophages did not migrate in response to MCP-1. In contrast, IL-2 enhanced the expression of CCR2 and augmented the chemotactic capacity of resting natural killer cells and T lymphocytes (22).

The non-redundant role of CCR2 in monocyte recruitment and host defense has been demonstrated in mice with a targeted disruption of the CCR2 gene (23, 24). Monocytes from CCR2-deficient mice did not migrate in response to MCP-1, and the mice failed to recruit monocytes in an experimental inflammation model and to clear infection. Furthermore, the monocytes from the CCR2-deficient mice were less able to adhere to the endothelium, suggesting that CCR2 is not only involved in chemotaxis but may also support agonist-induced firm adhesion of monocytes to the endothelium. This hypothesis was confirmed by recent findings that have demonstrated that the stimulation of chemoattractant receptors by their respective agonists can trigger the integrin-dependent adhesion of leukocytes to vascular ligands (25).

In previous reports we described the inhibitory effects of pro-inflammatory cytokines on monocyte CCR2 expression in vitro, which contrasted the effects of LDL (21, 26). In the present study we investigated monocyte CCR2 expression in vivo. Our data suggest a regulatory role for plasma lipoproteins, and we demonstrate an enhanced CCR2 expression in monocytes isolated from hypercholesterolemic patients. High density lipoprotein (HDL) can prevent and even reverse the effects of LDL. Our results support the hypothesis that pro-atherogenic lipoproteins enhance the chemotactic motility that may contribute to increased recruitment of monocytes to the vessel wall and accelerate the events of atherogenesis.

MATERIALS AND METHODS

Subjects

Forty-eight post-menopausal women (at least 6 months since onset) with various plasma lipid levels participated in this study. Informed consent was obtained from all subjects and the experimental protocol was approved by the Human Subjects Committee. The participants were nonsmokers and had no clinical evidence of diseases. Twenty women were selected and subdivided into four categories according to their plasma LDL cholesterol (LDL-C) and HDL cholesterol (HDL-C) levels. Group A contained 6 individuals with LDL-C$<$100 mg/dl and HDL-C$\geqslant$50 mg/dl; group B included 4 subjects with LDL-C$<$100 mg/dl and HDL-C$<$40 mg/dl; group C included 6 individuals with LDL-C$\geqslant$160 mg/dl and HDL-C$\geqslant$50 mg/dl; and group D included 4 participants with LDL-C$\geqslant$160 mg/dl and HDL-C$<$40 mg/dl (Table 1).

Another group of 28 volunteers received estrogen supplement (0.625 mg of 17-β estradiol p.o. per day). Their lipid profile was determined prior to the treatment, and the subjects were divided into three groups according to their LDL-C/HDL-C ratios. The plasma level of 17-β estradiol was monitored regularly using the Biodata radioimmune assay kit (Biodata Diagnostic, New York, NY). The replacement therapy increased the estrogen level from 15.0 ± 26.5 pg/ml to 54.4 ± 65.8 pg/ml ($P < 0.01$ by paired *t*-test). After 2 months on therapy the final analyses were performed.

Isolation of human monocytes

For the isolation of human monocytes the subjects were instructed to fast overnight and 20 ml of blood was drawn into tubes containing 3 mm EDTA. The buffy coat was isolated by centrifugation (1500 *g* for 30 min at 4°C), and 7 ml of the buffy coat and adjacent plasma was carefully layered onto 3 ml of Histopaque, d 1.077 g/ml (Sigma, St. Louis, MO). The leukocytes were separated by centrifugation (400 *g* for 30 min at 22°C). To further purify the monocytes and to eliminate platelets, the cells were washed twice with PBS containing 0.1% BSA and 0.02% EDTA. The cells were recovered by centrifugation (400 *g* for 15 min at 4°C), plated for 2 h at 37°C in RPMI-1640 medium (Life Technologies, Inc., Gaithersburg, MD) and 10% autologous serum, and the adhering cells were harvested for RNA preparation. The purity of monocytes was >90% estimated by flow cytometry using anti CD14 antibody (Pharmingen, San Diego, CA).

Cell culture and incubation conditions

THP-1 monocytes were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in RPMI 1640 containing low endotoxin calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin (Gemini Bioproducts Inc., Calabasas, CA), and 20 μm mercaptoethanol. To prevent differentiation and down-regulation of CCR2, the cell concentration was always kept below 0.5×10^6 cells/ml. The incubations with various concentrations of LDL, HDL, and apolipoprotein A-I were carried out in 5% lipoprotein-deficient serum. For the treatment of the cells with 17-β estradiol, medium supplemented with 1% serum was used to minimize the effects of 17-β estradiol contained in the serum, which was 0.08 ± 0.02 pg/ml ($<10^{-12}$ m) according to the specification of the distributor (Gemini Bioproducts Inc., Calabasas, CA). After the treatment, the cells were washed three times with PBS and used for the analyses as described. Cell viability, determined by trypan blue exclusion, was greater than 95%. The endotoxin level in the incubation medium was routinely monitored by the timed gel formation method using the endotoxin kit (Sigma, St. Louis, MO) and never exceeded 0.05 ng/ml, even at the highest lipoprotein concentrations used. At this concentration endotoxin does not affect monocyte CCR2 expression (27).

Preparation of lipoproteins

Lipoprotein fractions were prepared by ultracentrifugation as described (28). Native human LDL (d 1.03–1.063 g/ml) and HDL (d 1.063–1.21 g/ml) were isolated from fresh plasma of normolipidemic donors and stored under N_2 in the dark at 4°C in PBS containing 1 mm EDTA. The subfractions of HDL, HDL_2

(d 1.063–1.12 g/ml) and HDL_3 (d 1.15–1.21 g/ml) were prepared from HDL by serial centrifugation. For the preparation of apolipoprotein A-I, lyophilized HDL was delipidated with ethanol–ether 3:2 (v/v); apolipoprotein A-I was purified by anion exchange chromatography on Q-Sepharose (29); and the concentration of apolipoprotein A-I was adjusted to 5 mg/ml PBS. The integrity of the lipoproteins was confirmed by agarose gel electrophoresis as described (30) and the preparations showed no evidence of oxidation (thiobarbituric acid-reactive substances <0.5 nmol/mg protein).

Analysis of CCR2 transcripts

Total RNA was isolated by guanidinium thiocyanate–phenol–chloroform extraction (31). The mRNA was reverse transcribed from 2 μg total RNA using 0.12 nmol oligo(dT)10 primers (Roche Molecular Biochemicals, Indianapolis, IN) and Superscript II (Life Technologies, Inc., Gaithersburg, MD). CCR2 gene expression was then estimated by semi-quantitative PCR using a sense primer (5′-ATGCTGTCCACATCTCGTTCTCG) and an anti-sense primer (5′-TTATAAACCAGCCGAGACTTCCTGC) to yield full-length CCR2B (1083 base pairs) as described (26). Typically, the amplification was carried out for 30 cycles with an annealing temperature of 62°C. The concentration of the reverse transcribed cDNA in the PCR mixture was adjusted to assure a linear correlation between template and product. The specificity of the amplification was confirmed by DNA sequencing, and all samples tested negative for contamination with genomic DNA in PCR analyses of RNA samples without prior treatment with reverse transcriptase. The amplified DNA was analyzed by agarose gel electrophoresis, the intensity of the band stained with ethidium bromide was estimated by densiometric scanning (Image Quant, Molecular Dynamics, Sunnyvale, CA) and compared to that of the internal standard. As internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified under identical conditions as described (26), using 5′-TCGGAGTCAAC GGATTTGGTCGTA as sense primer and 5′-ATGGACTGTGGTC ATGAGTCCTTC as anti-sense primer.

Equilibrium binding analysis

THP-1 monocytes incubated with various concentrations of HDL were isolated by centrifugation, resuspended at 3×10^6 cells/ml in 100 μl RPMI 1640 without phenol red (Irvine Scientific, Santa Ana, CA) containing 0.5% BSA and 20 mm HEPES, pH 7.4, and the binding analysis was carried out essentially as described (21). Briefly, the suspended cells were incubated for 90 min at room temperature with 0.05 nm of ^{125}I-labeled MCP-1 (2200 Ci/mmol, DuPont NEN, Boston, MA) in the absence and presence of 50 nm unlabeled MCP-1. The cells were separated from the incubation mix by centrifugation through dibutylphthalate–dioctylphthalate 1:1 (v/v) and the ^{125}I-labeled MCP-1 associated with the cells was estimated by counting the radioactivity in a gamma counter. The specific binding was calculated by subtracting the nonspecific binding estimated in the presence of unlabeled MCP-1 from the total binding.

Chemotaxis assay

THP-1 monocytes were suspended at a concentration of 2×10^6 cells/ml in chemotaxis buffer consisting of RPMI 1640 without phenol red, 0.2% BSA, and 20 mm HEPES (pH 7.4). The cell suspension (51 μl) was loaded in the upper chamber of the 48-well microchemotaxis chamber, and MCP-1 was added to the lower chamber which was separated from the upper chamber by a 5-μm polycarbonate membrane. After 120 min of incubation at 37°C in a 5% CO_2 atmosphere, the side of the membrane that was in contact with the cell suspension was scraped off to remove any cells; the THP-1 monocytes attached to the underside of the membrane were fixed in 1% glutaraldehyde and stained with crystal violet. The number of cells that had migrated through the membrane was determined by counting the cells in four high power 400× fields per well under a microscope. Each experiment was done in triplicate. All results were expressed as migration index defined as the number of cells migrating in response to chemoattractant divided by the number of cells migrating in response to buffer.

Determination of cellular and plasma lipids

For the estimation of cellular cholesterol, lipids were extracted from 1×10^6 cells with chloroform–methanol–HCL (0.03 m) 2:1:1 (v/v/v), dried under N_2, and dissolved in 50 μl isopropanol. Free cholesterol and total cholesterol, obtained after hydrolysis of cholesteryl ester with cholesterol hydrolase, were estimated by the fluorimetric method and the content of cholesteryl ester was calculated by subtracting free cholesterol from total cholesterol (32). Total plasma triglycerides, total cholesterol, and HDL cholesterol after precipitation of other lipoproteins with heparin/manganese (33), were measured by enzymatic techniques (Boehringer Mannheim, Indianapolis, IN) with an Hitachi 7150 automatic analyzer (Hitachi, Japan).

Other analytical procedures

Protein was determined by the method of Lowry et al. (34). Lipoproteins were tested for oxidation by the fluorimetric assay for thiobarbituric acid-reactive substances (35). All assays were done in triplicate and the statistical analyses of the data were performed using the SPSS-PC program package (SPSS Inc.). All data were expressed as mean ± standard deviation (SD).

RESULTS

Correlation between plasma lipids and CCR2 expression in circulating monocytes

The effect of plasma lipids on CCR2 expression in circulating monocytes was studied on 20 post-menopausal, healthy, non-smoking females matched for age. **Table 1** summarizes the lipid profiles of the volunteers. They were selected and subdivided into four groups according to their LDL-C and HDL-C levels. The individuals with low LDL-C were assigned to groups A and B, and the volunteers with high LDL-C levels were placed in groups C and D. Groups A and B displayed no significant difference in their low LDL-C levels (<100 mg/dl), but the HDL-C level was significantly lower in group B compared to A. In contrast, groups C and D showed LDL-C levels characteristic for hypercholesterolemia (>160 mg/dl), with high HDL-C levels in group C and low HDL-C levels in group D. Circulating monocytes were isolated from these subjects and the expression of CCR2 was estimated by semi-quantitative RT-PCR. As internal standard, the expression of GAPDH was analyzed under identical conditions. As shown in Table 1 and in **Fig. 1**, the monocyte CCR2 expression in the high LDL-C group was at least 2-fold higher compared to the normolipidemic control group, provided the HDL-C level was low (group D vs. B). Of considerable interest, there was a significant inverse correlation between CCR2 expression and HDL-C, but only in the high LDL-C groups C and D (Spearman Correlation: $r = -0.6168$, $P = 0.029$). In contrast, basal CCR2 expres-

TABLE 1. Plasma lipid profile and monocyte CCR2 expression in normo- and hypercholesterolemic women

	Group				
	A (n = 6)	B (n = 4)	C (n = 6)	D (n = 4)	ANOVA (*P* value)
Age (yr)	46.7 ± 4.2	53.0 ± 6.3	53.5 ± 6.3	55.8 ± 7.3	NS
BMI (kg/m^2)	22.5 ± 1.0	23.7 ± 3.0	23.0 ± 2.5	24.5 ± 3.5	NS
TC (mg/dl)	167.3 ± 13.0	153.8 ± 42.1	270.5 ± 25.9	258.0 ± 18.9	<0.01
TG (mg/dl)	109.3 ± 38.3	266.4 ± 148.2	92.2 ± 20.3	173.8 ± 81.5	NS
HDL-C (mg/dl)	67.2 ± 11.4	35.8 ± 7.1	62.3 ± 2.7	37.2 ± 2.9	<0.01
LDL-C (mg/dl)	78.2 ± 12.7	64.7 ± 21.7	189.7 ± 24.0	186.0 ± 25.9	<0.01
LDL-C/HDL-C	1.2 ± 0.3	1.9 ± 0.8	3.0 ± 0.4	5.0 ± 0.6	<0.001
CCR2 (% of GAPDH)	64 ± 48	52 ± 35	72 ± 35	127 ± 42	<0.05

The subjects were divided into 4 groups according to their plasma LDL-C and HDL-C (group A: low LDL-C, high HDL-C; group B: low LDL-C, low HDL-C; group C: high LDL-C, high HDL-C; group D: high LDL-C, low HDL-C). Circulating monocytes were isolated and the CCR2 expression was estimated by semi-quantitative RT-PCR as described. BMI, body mass index; TC, total cholesterol; TG, triglycerides; NS, not significant ($P \geq 0.05$); CCR2, monocyte CCR2 mRNA normalized to GAPDH.

Statistical analysis: Kruskal-Wallis one-way ANOVA by ranks.

sion was unaffected by HDL in the low LDL-C groups (groups A and B). Similarly, triglycerides did not affect CCR2 expression.

Reduction of monocyte CCR2 expression by modulating plasma LDL and HDL levels

To study the effects of lipoproteins more directly, we sought to modulate lipid profiles and to assess whether changes of plasma LDL and HDL levels affect CCR2 expression in circulating monocytes in vivo. We had available to us additional 28 postmenopausal women who were placed on estrogen replacement therapy. Their lipid parameters and levels of monocyte CCR2 expression were determined prior to the start of the therapy and are compiled in **Table 2**. Based on the results summarized in Table 1 suggesting that LDL directly and HDL inversely affected CCR2 expression, the volunteers were divided into three groups according to their LDL/HDL cholesterol ratios. Group A represented the control (basal LDL/HDL cholesterol ratio prior to estrogen therapy <3), group B contained hypercholesterolemic individuals, but with high basal HDL-C (LDL/HDL cholesterol ratio between 3 and 4), and group C represented hypercholesterolemic individuals with low HDL-C (LDL/HDL cholesterol ratio >4). For the group as a whole, the CCR2 expression, determined by RT-PCR and normalized to GAPDH, correlated well with plasma LDL-C levels (Spearman correlation: $r = 0.339$, $P = 0.039$), LDL/HDL cholesterol ratios (Spearman correlation: $r = 0.359$, $P = 0.019$) and total cholesterol levels (Spearman correlation: $r = 0.379$, $P = 0.021$). Analysis of the results from the whole study (n = 48) clearly confirmed the correlation between monocyte CCR2 expression and the plasma lipid levels (**Table 3**). The most significant correlation was seen between monocyte CCR2 expression and the LDL/HDL cholesterol ratios (**Fig. 2**).

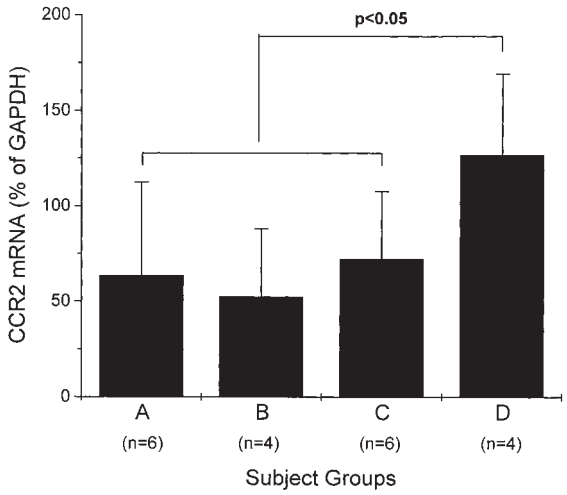

Fig. 1. The effect of plasma lipoproteins on the CCR2 expression in circulating human monocytes. Twenty nonsmoking female volunteers were divided into four groups according to their plasma LDL-C and HDL-C levels as described in Table 1 (group A: LDL-C<100 mg/dl, HDL-C≥50 mg/dl; group B: LDL-C<100 mg/dl, HDL-C<40 mg/dl; group C: LDL-C≥160 mg/dl, HDL-C≥50 mg/dl; group D: LDL-C≥160 mg/dl, HDL-C<40 mg/dl). Circulating monocytes were isolated, total RNA was prepared, and CCR2 mRNA was estimated by semi-quantitative RT-PCR as described under Materials and Methods. The results are expressed as percent (mean ± SD) of GAPDH mRNA, estimated under identical conditions, and monocyte CCR2 expression among the four groups was first analyzed by the non-parametric Kruskal-Wallis one-way ANOVA by ranks. The significance of the differences between monocyte CCR2 expression in group D versus all other groups was then established by the non-parametric Mann-Whitney U test ($P < 0.05$).

It is well established that estrogen supplement decreases plasma LDL-C and increases HDL-C in menopausal women (36). Therefore, the lipid profiles and monocyte CCR2 expression were determined again 2 months after the women were placed on estrogen (0.625 mg per day), and the results are shown in the lower section of Table 2. In the control group A (basal LDL/HDL cholesterol ratio <3), estrogen treatment produced no significant changes in either lipoprotein profile or CCR2 expression. In the hypercholesterolemic group C with low basal HDL-C (LDL/HDL cholesterol ratio >4), the estrogen treatment decreased plasma LDL-C, increased plasma HDL-C, and improved LDL/HDL cholesterol ratios. Fur-

TABLE 2. Effect of estrogen replacement therapy on plasma lipids and monocyte CCR2 expression in postmenopausal women

	Group			
	LDL/HDL<3 (n = 11)	3≤LDL/HDL≤4 (n = 11)	LDL/HDL>4 (n = 6)	ANOVA (*P* value)
Age (yr)	51.3 ± 6.5	55.5 ± 4.4	55.3 ± 5.7	NS
BMI (kg/m^2)	22.9 ± 2.0	24.5 ± 2.6	25.3 ± 3.0	NS
Before treatment				
TC (mg/dl)	198.8 ± 42.2	256.5 ± 29.2	255.0 ± 22.4	<0.001
TG (mg/dl)	132.3 ± 72.5	151.5 ± 48.1	207.2 ± 74.8	NS
HDL-C (mg/dl)	60.3 ± 15.2	51.4 ± 7.9	38.2 ± 2.7	<0.01
LDL-C (mg/dl)	112.1 ± 46.5	174.8 ± 27.1	175.2 ± 22.9	<0.01
LDL-C/HDL-C	2.0 ± 0.8	3.4 ± 0.3	4.6 ± 0.7	<0.0001
CCR2 (% of GAPDH)	69 ± 39	97 ± 42	118 ± 34	<0.05
After treatment				
TC (mg/dl)	195.8 ± 33.4	232.2 ± 27.2	242.2 ± 21.1	<0.01
TG (mg/dl)	132.9 ± 66.1	167.2 ± 79.8	238.0 ± 179.7	NS
HDL-C (mg/dl)	61.4 ± 11.0	58.9 ± 13.5	50.7 ± 9.8[a]	NS
LDL-C (mg/dl)	107.8 ± 35.3	139.9 ± 27.6[a]	143.9 ± 44.9	0.05
LDL-C/HDL-C	1.8 ± 0.6	2.5 ± 0.7[b]	2.9 ± 1.1[b]	<0.05
CCR2 (% of GAPDH)	64 ± 41	77 ± 29	78 ± 25[a]	NS

Twenty-eight postmenopausal women were placed on estrogen therapy (0.625 mg/day), and their plasma lipid profile and monocyte CCR2 expression were determined at the start of the treatment and again after 2 months. They were divided into 3 groups according to their LDL-C/HDL-C ratios prior to the treatment. LDL/HDL indicates the ratio between LDL-C and HDL-C; BMI, body mass index; TC, total cholesterol; TG, triglycerides; NS, not significant ($P \geq 0.05$); CCR2, monocyte CCR2 mRNA normalized to GAPDH. Statistical analysis: Kruskal-Wallis one-way ANOVA by ranks.

[a] $P < 0.05$ and [b] $P < 0.01$ versus before treatment by paired *t*-test.

ther, monocyte CCR2 expression was reduced almost 2-fold ($P = 0.028$) and was then comparable to the control group (**Fig. 3A**, Table 2). In the group B with a basal LDL/HDL cholesterol ratio between 3 and 4, estrogen lowered LDL-C significantly and increased HDL-C slightly, which significantly decreased the LDL/HDL ratio. The treatment also lowered monocyte CCR2 expression, although not statistically significant. Multiple regression analysis indicated that the change in the LDL/HDL cholesterol ratios in the hypercholesterolemic patients by the estrogen treatment was the most significant factor to predict a change in monocyte CCR2 expression (Significant T = 0.003). No significant correlation between the individual changes of LDL-C or HDL-C and changes in CCR2 expression was found. In contrast, a highly significant correlation was found between the changes of LDL/HDL cholesterol ratios and the changes in CCR2 expression (Fig. 3B). These findings are consistent with our hypothesis that monocyte CCR2 expression and chemotactic response to MCP-1 are in part regulated by plasma lipoproteins.

To test whether estrogen directly affected CCR2 expression, we incubated THP-1 monocytes with various concentrations of 17-β estradiol (10^{-10} to 10^{-8} m) for 24 h and then estimated CCR2 expression by semi-quantitative RT-PCR. The results demonstrated that 17-β estradiol affected neither the CCR2 mRNA level nor the chemotactic response to MCP-1 (data not shown), indicating that the reduction in monocyte CCR2 expression after estrogen

TABLE 3. Correlation between demographic and lipid parameters and monocyte CCR2 expression

	r	*P*
Age (yr)	0.261	0.074
BMI (kg/m^2)	0.225	0.125
TC (mg/dl)	0.380	0.008
TG (mg/dl)	0.186	0.205
HDL-C (mg/dl)	0.225	0.125
LDL-C (mg/dl)	0.375	0.009
LDL-C/HDL-C	0.441	0.002

Data were compiled from the results of the analyses of the entire study population (n = 48). The correlation between the individual parameters and monocyte CCR2 expression was determined by the Spearman analysis.

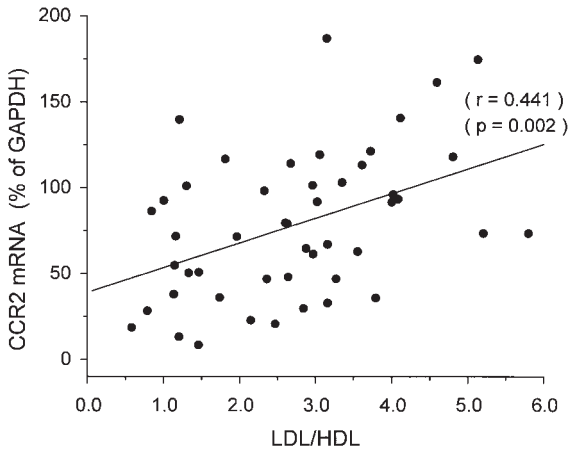

Fig. 2. Correlation between monocyte CCR2 expression and plasma LDLC/HDL-C ratios. The CCR2 mRNA data of all subjects in the study (n = 48) were blotted as a function of the respective plasma LDL and HDL cholesterol ratios (LDL/HDL) and the regression line was calculated (Spearman analysis, $r = 0.441$; $P = 0.002$).

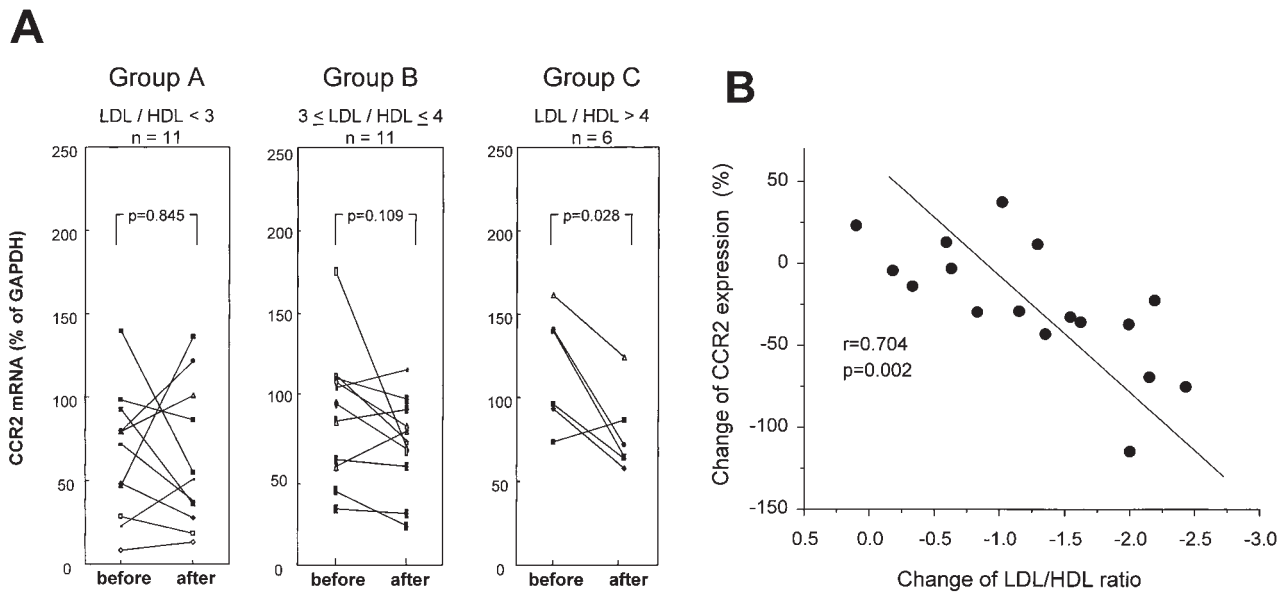

Fig. 3. Effect of lowering plasma LDL cholesterol and increasing plasma HDL cholesterol on monocyte CCR2 expression. (A) The lipid profile and monocyte CCR2 expression was determined on 28 postmenopausal women before and after (2 months) estrogen replacement therapy (0.625 mg p.o. per day). The volunteers were divided into three groups according to their pre-therapy plasma LDL-C / HDL-C ratios (group A: LDL-C / HDL-C<3; group B: 3≤LDL-C / HDL-C≥4; group C: LDL-C / HDL-C>4). Monocyte CCR2 expression was determined by semi-quantitative RT-PCR as described in Fig. 1 and the expression levels before (before) and after (after) estrogen replacement therapy were compared. The data were analyzed by the paired *t*-test; n, number of subjects. (B) Correlation between the changes in the LDL / HDL cholesterol ratios and the changes in monocyte CCR2 expression. The changes in the levels of CCR2 expression were blotted as a function of the calculated differences in the LDL / HDL cholesterol ratios of the hypercholesterolemic patients (group B and C; n = 17) before and after the estrogen replacement therapy. The correlation was determined by the Spearman analysis ($r = 0.704$; $P = 0.002$).

replacement therapy was mainly due to the elicited changes in the lipoprotein profiles.

LDL enhances MCP-1 mediated chemotaxis in THP-1 monocytes

The physiological role of increased CCR2 expression was tested by chemotaxis assays. The results from our previous study indicated that LDL substantially increased the expression of CCR2 in THP-1 monocytes (26). To determine the effect on the chemotactic activity, THP-1 monocytes were incubated with LDL (50 μg of protein / ml) for 24 h. This treatment increased both CCR2 mRNA and protein about 2-fold (data not shown), resulting in an enhanced chemotactic response to MCP-1 (**Fig. 4**). MCP-1 induced chemotaxis in untreated THP-1 monocytes with a maximal response between 2 and 10 nm MCP-1 (Fig. 4), which was comparable to that of human blood monocytes (37). Treatment of the cells with LDL increased the chemotaxis elicited by MCP-1 about 2 fold at optimal (10 nm) concentration of the chemoattractant.

Reduction of CCR2 expression by HDL

The results from the in vivo experiments suggested that CCR2 expression in circulating monocytes was affected by LDL as well as by HDL. In a recent study we have demonstrated that a cellular increase of LDL-derived cholesterol substantially induced monocyte CCR2 expression (26). Conversely, any mechanism resulting in a reduction in cellular cholesterol should lower CCR2 expression. We therefore postulated that HDL particles might act directly on circulating monocytes and decrease CCR2 expression by mediating cholesterol efflux. To test this hypothesis we incubated THP-1 monocytes for 24 h with various concentrations of HDL ranging from 0 to 300 μg of HDL protein / ml. For the duration of the experiments, the cells were kept in medium

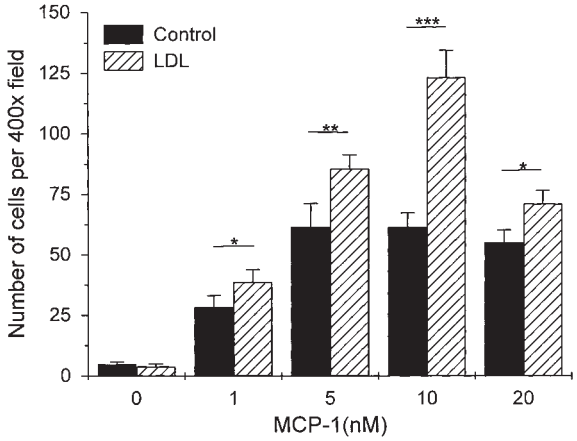

Fig. 4. Effect of LDL on monocyte chemotaxis. THP-1 monocytes were treated with native LDL (50 μg protein / ml) in 5% lipoprotein-deficient serum for 24 h. Chemotaxis in response to the indicated concentrations of MCP-1 was estimated in a Boyden chamber as described under Materials and Methods. The number of migrated cells was determined by counting the cells in four random high power 400× fields and expressed as the mean number of cells per field. Data represent the mean ± SD of three independent experiments and were analyzed by *t*-test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$.

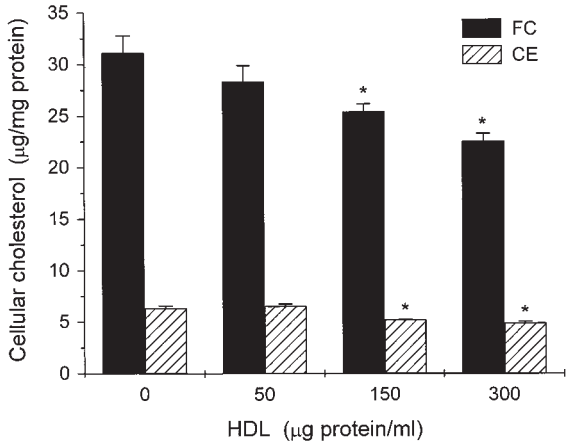

Fig. 5. Cholesterol efflux from THP-1 monocytes to HDL. THP-1 monocytes were incubated for 24 h with the indicated concentrations of HDL in 5% lipoprotein-deficient serum. At the end of the incubation period, the cells were collected and the content of free cholesterol (FC) and cholesteryl ester (CE) was determined. The data are expressed as the mean ± SD of three independent experiments and were analyzed by *t*-test. $*P < 0.05$ versus the untreated cells.

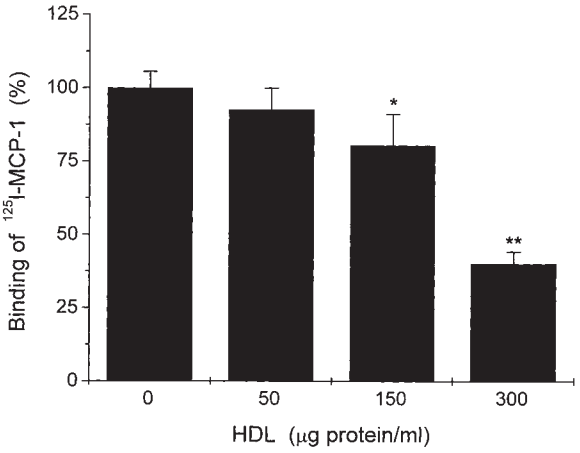

Fig. 7. Reduction of functional CCR2 protein in THP-1 monocytes treated with HDL. THP-1 monocytes were treated with HDL as described in Fig. 5 and the effect on the expression of functional CCR2 protein on the cell surface was analyzed by ligand binding assay as described under Materials and Methods. Shown is the specific binding of ^{125}I-labeled MCP-1 estimated by subtracting nonspecific binding, determined in the presence of an excess of unlabeled MCP-1, from the total binding. Data are expressed as percent specific binding of that of untreated control cells (100%), which bound 3.8 ± 0.2 fmol ^{125}I-labeled MCP-1/10^6 cells. The data represent the mean ± SD of three independent experiments and were analyzed by *t*-test. $*P < 0.05$; $**P < 0.001$ versus untreated control cells.

supplemented with 5% lipoprotein-deficient human serum to prevent any effects mediated by lipoproteins of the calf serum. The HDL preparations used in our experiments showed no evidence of oxidation (thiobarbituric acid-reactive substances <0.5 nmol/mg protein), and the endotoxin levels in the incubation medium even at the highest HDL levels never exceeded 0.05 ng/ml and did not affect CCR2 expression (27). Incubation of THP-1 monocytes with HDL for 24 h resulted in a dose-dependent decrease of intracellular cholesterol (**Fig. 5**). The reduction of cellular cholesterol was paralleled by a concomitant decrease of CCR2 transcripts (**Fig. 6**). The whole particle was most effective although the subfractions HDL_2 and HDL_3 also significantly reduced CCR2 expression. The inhibition of CCR2 gene expression by HDL was also observed in the presence of LDL, provided that the LDL/HDL cholesterol ratio <3 (data not shown).

The change in CCR2 protein was estimated by ^{125}I-labeled MCP-1 binding analysis and correlated well with the respective changes of the mRNA levels (**Fig. 7**), sug-

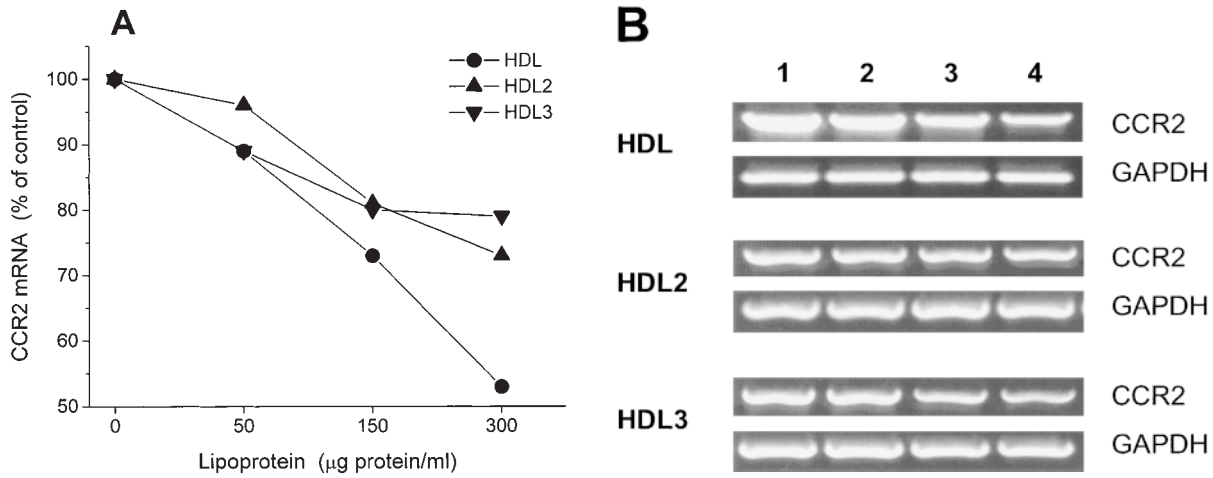

Fig. 6. Inhibition of monocyte CCR2 expression by HDL. THP-1 monocytes cultured in 5% lipoprotein-deficient serum were incubated for 24 h with the indicated concentrations of either HDL or with the subfractions HDL_2 or HDL_3. (A) CCR2 expression was determined by semi-quantitative RT-PCR, normalized to GAPDH estimated under identical conditions, and expressed as percent of that of untreated control cells. (B) Agarose gel electrophoresis of amplified CCR2 transcripts of THP-1 cells after treatment with HDL, HDL_2 or HDL_3. Lane 1, untreated cells; lanes 2–4, cells treated with HDL at 50, 150, and 300 μg protein/ml.

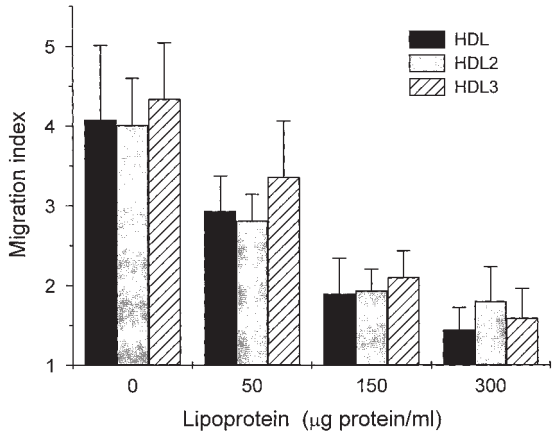

Fig. 8. Effect of HDL-induced inhibition of CCR2 expression on monocyte chemotaxis. For the final 24 h before analysis, THP-1 monocytes were incubated in 5% lipoprotein-deficient serum with the indicated concentrations of either HDL or the subfractions HDL_2 or HDL_3. At the end of the treatment, the cells were isolated by centrifugation and suspended in chemotaxis buffer. The cells were then placed into the upper chamber of the Boyden chamber and the migration rate induced by MCP-1 (10 nm) was determined as described under Materials and Methods. The number of migrated cells was counted under a microscope in four random high power 400× fields and averaged. The data are expressed as migration index and represent the mean ± SD of three independent experiments. The migration index is defined as the number of cells migrating in response to chemoattractant divided by the number of cells migrating in response to buffer.

gesting that HDL regulates CCR2 expression at the level of gene transcription. The reduction of CCR2 had a profound effect on chemotaxis. A loss of about 60% of surface receptors achieved by incubation of THP-1 monocytes with HDL at 300 μg protein/ml greatly reduced the chemotactic response of THP-1 monocytes to 10 nm MCP-1 (**Fig. 8**).

Finally, we determined whether the reduction in CCR2 expression by HDL was induced by receptor-mediated signaling. Recently, a murine and a human receptor for HDL were identified and shown to be expressed at high levels in circulating monocytes (38, 39). To test whether receptor-mediated transmembrane signaling reduced CCR2 expression, we incubated THP-1 monocytes with delipidated apolipoprotein A-I which can bind directly to the scavenger receptor BI (40) but mediates cholesterol efflux very inefficiently (41). Incubation of THP-1 monocytes for 24 h with various concentrations of apolipoprotein A-I did not change the cellular cholesterol levels (**Fig. 9A**) and did not affect MCP-1-mediated chemotaxis, indicating unaltered CCR2 expression (Fig. 9B). These results suggested that CCR2 expression was not regulated by lipoprotein-mediated and receptor-dependent transmembrane signaling, but was primarily affected by cellular cholesterol levels.

DISCUSSION

In these studies we examined the role of plasma lipoproteins on CCR2 expression and monocyte function. Monocyte recruitment from the circulation to the intima and the subendothelial accumulation of monocyte-derived macrophages are characteristic events in the initiation and progression of atherosclerosis. These cells ingest massive amounts of lipids and contribute to the formation and propagation of atherosclerotic lesions (1–3). Further, lesion-derived foam cells were shown to constitutively express matrix metalloproteinases that degrade components of the extracellular matrix and weaken atherosclerotic tissue at certain macrophage-rich areas resulting in plaque rupture (42). Thus, cells of the monocytic lineage may not only be involved in the events leading to plaque initia-

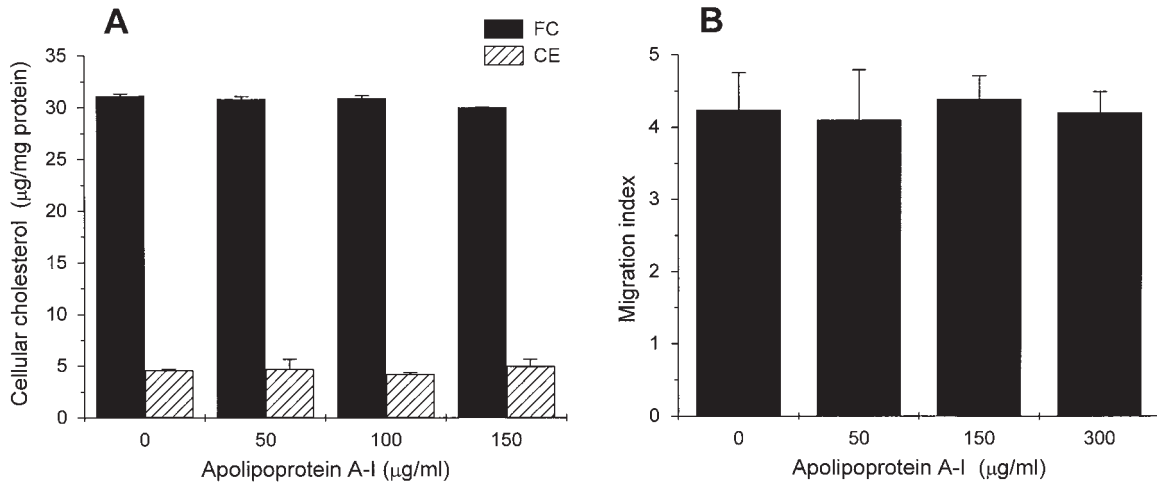

Fig. 9. Effect of apolipoprotein A-I on cellular cholesterol and monocyte chemotaxis. THP-1 monocytes were incubated for 24 h with the indicated concentrations of delipidated apolipoprotein A-I in 5% lipoprotein-deficient serum. At the end of the incubation period, the cells were collected by centrifugation and (A) the cellular content of free cholesterol (FC) and of cholesteryl ester (CE), and (B) the chemotaxis mediated by 10 nm MCP-1 were determined. Chemotaxis was expressed as migration index as described in Fig. 8. All values are expressed as the mean ± SD of three independent experiments.

tion and progression, but could also play an important function in the acute clinical events of atherosclerosis.

Endothelial cell dysfunction and changes in gene expression have been extensively associated with hypercholesterolemia (43). Conversely, a growing body of evidence suggests that lipoproteins may also affect gene expression in monocytes. Monocytes from hypercholesterolemic subjects or animal models were shown to be functionally different, they were more responsive to inflammatory stimuli and adhered in larger numbers to the endothelium compared with monocytes from normocholesterolemic controls (44–47). The non-redundant role of CCR2 in monocyte chemotaxis and recruitment to the arterial wall during atherogenesis has recently been demonstrated in mouse models susceptible to atherosclerosis. The targeted disruption of the CCR2 gene in apolipoprotein $E^{-/-}$ mice resulted in a considerable reduction of atherosclerotic lesion formation promoted by high fat diet (48). Similarly, the disruption of the MCP-1 gene in LDL receptor$^{-/-}$ mice reduced monocyte recruitment to the vessel wall and lipid deposition in the aorta (49). Conversely, up-regulation of monocyte CCR2 expression may accelerate monocyte accumulation in the vessel wall and increase lesion formation. In a previous report we demonstrated that CCR2 expression in THP-1 monocytes was greatly affected by LDL (26). In this in vivo study we provide evidence that monocytes isolated from hypercholesterolemic patients showed an about 2-fold increase in CCR2 expression. There was a significant correlation between plasma LDL levels and monocyte CCR2 expression, but only in individuals with low HDL levels. HDL was identified as a lipid parameter that inversely affected the expression of CCR2, and high levels of HDL (HDL-C $>$ 50 mg/dl) significantly reduced the LDL-induced expression of CCR2 in hypercholesterolemic subjects. In contrast, monocyte CCR2 biosynthesis was not affected by triglycerides.

Although the exact mechanisms by which lipoproteins affect CCR2 gene expression remain unclear, intracellular cholesterol appears to play a regulatory function. Increasing cellular cholesterol by incubation of monocytes with either LDL or free cholesterol resulted in enhanced CCR2 expression (26). Accordingly, lowering cellular cholesterol should reduce CCR2 expression. HDL plays a critical role in the reverse cholesterol transport, i.e., transport from the peripheral tissue to the liver (50). A murine receptor with high affinity for HDL termed scavenger receptor BI was recently identified (38). This receptor is thought to play an important function in the selective uptake of cholesteryl esters from HDL, and it also mediates cellular cholesterol efflux (51). In a previous study we have shown that the human homologue of scavenger receptor BI was present on circulating monocytes and THP-1 cells (39). Although the function of this receptor on monocytes remains unclear, it could play a role in the maintenance of cholesterol homeostasis and mediate cholesterol efflux. This would lower cellular cholesterol and decrease monocyte CCR2 expression, as was demonstrated in subjects with high plasma HDL levels. These findings were further confirmed in in vitro experiments using THP-1 monocytes. Treatment with HDL reduced the cellular cholesterol level and CCR2 expression, which suggests a causal relationship between cholesterol and CCR2 biosynthesis.

Alternatively, CCR2 expression could be regulated by receptor-mediated transmembrane signaling. To test this possibility we incubated THP-1 monocytes with delipidated apolipoprotein A-I. The apolipoproteins of HDL, either reconstituted into phospholipid vesicles or in the delipidated form, bind with high affinity to the scavenger receptor BI (40). However, in contrast to HDL, the purified apolipoproteins are very inefficient mediators of cholesterol efflux (41). The treatment of THP-1 monocytes with apolipoprotein A-I did not affect the cellular cholesterol content and the chemotactic response of the cells to MCP-1 remained unchanged, suggesting that CCR2 expression remained unchanged as well. These results indicated that the binding of HDL did not induce transmembrane signaling affecting CCR2 expression, and that HDL-mediated cholesterol efflux was necessary for the inhibition of CCR2 biosynthesis.

The differential effects of LDL and HDL on CCR2 expression were further tested in postmenopausal women before and after estrogen replacement therapy. Estrogen replacement therapy significantly altered the lipoprotein profile but not the levels of total plasma cholesterol. The therapy reduced plasma LDL cholesterol but increased HDL cholesterol. This trend was especially emphasized in the group with high LDL-C, low HDL-C, and an LDL/HDL cholesterol ratio $>$4 before the treatment. The therapy significantly improved the lipid profile, lowered the cholesterol ratio, and reduced monocyte CCR2 expression. The results suggested that the basal level of CCR2 expression in circulating monocytes is not entirely determined by the genetic predisposition of the individual, but may also be affected by the plasma lipid profile. Even short-term elevation of plasma LDL-C can increase monocyte CCR2 expression, but because of the relatively short half-life of monocytes in circulation, the expression level would most likely return to basal level within 48 h. Correspondingly, improvement of plasma lipid levels through cholesterol-lowering therapy will reduce monocyte CCR2 expression.

Enhanced CCR2 expression increased MCP-1–induced monocyte chemotaxis several fold, which may accelerate monocyte accumulation in the arterial wall in hypercholesterolemia. The increase in the chemotactic motility was particularly pronounced at chemoattractant concentrations that were optimal for chemotaxis. In contrast, HDL substantially inhibited monocyte CCR2 expression and reduced the chemotaxis mediated by MCP-1. In a previous study we demonstrated that the effects of lipoproteins on chemotaxis were specific for CCR2, and chemotaxis mediated by N-formylated peptides remained unchanged (26). This can, in part, be explained by the difference in the expression levels of the two chemoattractant receptors. On average, monocytes express between 50,000 and 100,000 receptor molecules for N-formylated peptides, but a full cellular response requires the activation of just a fraction

of the available receptors. In contrast, the level of monocyte stimulation by MCP-1 appears to be restricted by the rather low expression of CCR2 (about 5000 receptors per cell), and an optimal chemotactic response requires the activation of the entire receptor population. Any changes in CCR2 expression will, therefore, directly affect cellular function.

Leukocyte diapedesis is a complex process and involves reversible rolling, firm adhesion to the endothelium, and subsequent chemoattractant-mediated transendothelial migration (52). The initial adhesion is transient and involves the interaction between vascular selectins and monocyte ligands. This slows the movement of monocytes and may allow time to scan the vascular environment for chemoattractant signals. The subsequent firm integrin-mediated adhesion requires the activation of monocyte adhesion receptors and interaction with the endothelial counterreceptors. $\alpha 4\beta 1$ integrin has been identified as a potentially important monocyte adhesion receptor, and activation of chemoattractant receptors was shown to enhance the interaction between integrins and vascular cell adhesion molecule-1 which is expressed in a localized fashion in lesion-prone areas (25, 53, 54). Similarly, agonist-stimulated CCR2 may induce and enhance the activation-dependent adhesion of monocytes to the endothelium. Increased CCR2 expression may intensify this process, enhance endothelial transmigration and the chemotactic response to MCP-1, and magnify monocyte recruitment to the arterial wall during atherogenesis.

In summary, we have shown that monocyte gene expression is altered in hypercholesterolemia. High plasma concentrations of atherogenic LDL increase monocyte CCR2 expression and the chemotactic response to external stimuli. This may enhance monocyte recruitment to the intima and accelerate the formation of atherosclerotic lesions. In contrast, HDL helps to lower CCR2 expression and MCP-1–mediated chemotaxis, reducing monocyte accumulation in the arterial wall. Thus, the beneficial effects of lipid-lowering therapy on atherosclerosis may include the reduction of monocyte CCR2 expression and chemotactic motility to basal levels.

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REFERENCES

1. Gerrity, R. G. 1981. The role of the monocyte in atherogenesis: I. Transition of blood-borne monocytes into foam cells in fatty lesions. *Am. J. Pathol.* **103:** 181–190.
2. Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witztum. 1989. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320:** 915–924.
3. Ross, R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1980s. *Nature.* **362:** 801–809.
4. Faruqi, R. M., and P. E. DiCorleto. 1993. Mechanisms of monocyte recruitment and accumulation. *Br. Heart J.* **69:** S19–S29.
5. Rollins, B. J., E. D. Morrison, and C. D. Stiles. 1988. Cloning and expression of JE, a gene inducible by platelet-derived growth factor and whose product has cytokine-like properties. *Proc. Natl. Acad. Sci. USA.* **85:** 3738–3742.
6. Yoshimura, T., E. A. Robinson, S. Tanaka, E. Appella, J. Kuratsu, and E. J. Leonard. 1989. Purification and amino acid analysis of two human glioma-derived monocyte chemoattractants. *J. Exp. Med.* **169:** 1449–1459.
7. Carr, M. W., S. J. Roth, E. Luther, S. S. Rose, and T. A. Springer. 1994. Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. *Proc. Natl. Acad. Sci. USA.* **91:** 3652–3656.
8. Allavena, P., G. Bianchi, D. Zhou, J. Van Damme, P. Jílek, S. Sozzani, and A. Mantovani. 1994. Induction of natural killer cell migration by monocyte chemotactic protein-1, -2 and -3. *Eur. J. Immunol.* **24:** 3233–3236.
9. Hall, D. J., C. Brownlee, and C. D. Stiles. 1989. Interleukin-1 is a potent regulator of JE and KC gene expression in quiescent BALB/c fibroblasts. *J. Cell Physiol.* **141:** 154–159.
10. Rollins, B. J., T. Yoshimura, E. J. Leonard, and J. S. Pober. 1990. Cytokine-activated human endothelial cells synthesize and secrete a monocyte chemoattractant, MCP-1/JE. *Am. J. Pathol.* **136:** 1229–1233.
11. Luster, A. D. 1998. Chemokines—chemotactic cytokines that mediate inflammation. *N. Engl. J. Med.* **338:** 436–445.
12. Koch, A. E., S. L. Kunkel, L. A. Harlow, B. Johnson, H. L. Evanoff, G. K. Haines, M. D. Burdick, R. M. Pope, and R. M. Strieter. 1992. Enhanced production of monocyte chemoattractant protein-1 in rheumatoid arthritis. *J. Clin. Invest.* **90:** 772–779.
13. Yla-Herttuala, S., B. A. Lipton, M. E. Rosenfeld, T. Sarkioja, T. Yoshimura, E. J. Leonard, J. L. Witztum, and D. Steinberg. 1991. Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. *Proc. Natl. Acad. Sci. USA.* **88:** 5252–5256.
14. Cushing, S. D., J. A. Berliner, A. J. Valente, M. C. Territo, M. Navab, F. Parhami, R. Gerrity, C. J. Schwartz, and A. M. Fogelman. 1990. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc. Natl. Acad. Sci. USA.* **87:** 5134–5138.
15. Navab, M., S. S. Imes, S. Y. Hama, G. P. Hough, L. A. Ross, R. W. Bork, A. J. Valente, J. A. Berliner, D. C. Drinkwater, and H. Laks. 1991. Monocyte transmigration induced by modification of low density lipoprotein in cocultures of human aortic wall cells is due to induction of monocyte chemotactic protein 1 synthesis and is abolished by high density lipoprotein. *J. Clin. Invest.* **88:** 2039–2046.
16. Shyy, Y. J., L. L. Wickham, J. P. Hagan, H. J. Hsieh, Y. L. Hu, S. H. Telian, A. J. Valente, K. L. Sung, and S. Chien. 1993. Human monocyte colony-stimulating factor stimulates the gene expression of monocyte chemotactic protein-1 and increases the adhesion of monocytes to endothelial monolayers. *J. Clin. Invest.* **92:** 1745–1751.
17. Wenzel, U. O., B. Fouqueray, G. Grandaliano, Y. S. Kim, C. Karamitsos, A. J. Valente, and H. E. Abboud. 1995. Thrombin regulates expression of monocyte chemoattractant protein-1 in vascular smooth muscle cells. *Circ. Res.* **77:** 503–509.
18. Shyy, Y-J., H-J. Hsieh, S. Usami, and S. Chien. 1994. Fluid shear stress induces a biphasic response of human monocyte chemotactic protein 1 gene expression in vascular endothelium. *Proc. Natl. Acad. Sci. USA.* **91:** 4678–4682.
19. Charo, I. F., S. J. Myers, A. Herman, C. Franci, A. J. Connolly, and S. R. Coughlin. 1994. Molecular cloning and functional expression of two monocyte chemoattractant protein 1 receptors reveals alternative splicing of the carboxyl-terminal tails. *Proc. Natl. Acad. Sci. USA.* **91:** 2752–2756.
20. Wong, L. M., S. J. Myers, C. L. Tsou, J. Gosling, H. Arai, and I. F. Charo. 1997. Organization and differential expression of the human monocyte chemoattractant protein 1 receptor gene—Evidence for the role of the carboxyl-terminal tail in receptor trafficking. *J. Biol. Chem.* **272:** 1038–1045.
21. Tangirala, R. K., K. Murao, and O. Quehenberger. 1997. Regulation of expression of the human monocyte chemotactic protein-1 receptor (hCCR2) by cytokines. *J. Biol. Chem.* **272:** 8050–8056.

22. Loetscher, P., M. Seitz, M. Baggiolini, and B. Moser. 1996. Interleukin-2 regulates CC chemokine receptor expression and chemotactic responsiveness in T lymphocytes. *J. Exp. Med.* **184:** 569–577.
23. Boring, L., J. Gosling, S. W. Chensue, S. L. Kunkel, R. V. J. Farese, H. E. Broxmeyer, and I. F. Charo. 1997. Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *J. Clin. Invest.* **100:** 2552–2561.
24. Kuziel, W. A., S. J. Morgan, T. C. Dawson, S. Griffin, O. Smithies, K. Ley, and N. Maeda. 1997. Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. *Proc. Natl. Acad. Sci. USA.* **94:** 12053–12058.
25. Campbell, J. J., S. Qin, K. B. Bacon, C. R. Mackay, and E. C. Butcher. 1996. Biology of chemokine and classical chemoattractant receptors: differential requirements for adhesion-triggering versus chemotactic responses in lymphoid cells. *J. Cell Biol.* **134:** 255–266.
26. Han, K. H., R. K. Tangirala, S. R. Green, and O. Quehenberger. 1998. Chemokine receptor CCR2 expression and monocyte chemoattractant protein-1 mediated chemotaxis in human monocytes: a regulatory role for plasma low density lipoprotein. *Arterioscler. Thromb. Vasc. Biol.* **18:** 1983–1991.
27. Sica, A., A. Saccani, A. Borsatti, C. A. Power, T. N. Wells, W. Luini, N. Polentarutti, S. Sozzani, and A. Mantovani. 1997. Bacterial lipopolysaccharide rapidly inhibits expression of C-C chemokine receptors in human monocytes. *J. Exp. Med.* **185:** 969–974.
28. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
29. Weisweiler, P. 1987. Isolation and quantitation of apolipoproteins A-I and A-II from human high-density lipoproteins by fast-protein liquid chromatography. *Clin. Chim. Acta.* **169:** 249–254.
30. Yoshida, H., O. Quehenberger, N. Kondratenko, S. Green, and D. Steinberg. 1998. Minimally oxidized low-density lipoprotein increases expression of scavenger receptor A, CD36, and macrosialin in resident mouse peritoneal macrophages. *Arterioscler. Thromb. Vasc. Biol.* **18:** 794–802.
31. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162:** 156–159.
32. Heider, J. G., and R. L. Boyett. 1978. The picomole determination of free and total cholesterol in cells in culture. *J. Lipid Res.* **19:** 514–518.
33. Bachorik, P. S., P. D. Wood, J. J. Albers, P. Steiner, M. Dempsey, K. Kuba, R. Warnick, and L. Karlsson. 1976. Plasma high-density lipoprotein cholesterol concentrations determined after removal of other lipoproteins by heparin/manganese precipitation or by ultracentrifugation. *Clin. Chem.* **22:** 1828–1834.
34. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
35. Conti, M., P. C. Morand, P. Levillain, and A. Lemonnier. 1991. Improved fluorometric determination of malonaldehyde. *Clin. Chem.* **37:** 1273–1275.
36. Knopp, R. H., and X. Zhu. 1997. Multiple beneficial effects of estrogen on lipoprotein metabolism. *J. Clin. Endocrinol. Metab.* **82:** 3952–3954.
37. Yoshimura, T., and E. J. Leonard. 1990. Identification of high affinity receptors for human monocyte chemoattractant protein-1 on human monocytes. *J. Immunol.* **145:** 292–297.
38. Acton, S., A. Rigotti, K. T. Landschulz, S. Z. Xu, H. H. Hobbs, and M. Krieger. 1996. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science.* **271:** 518–520.
39. Murao, K., V. Terpstra, S. R. Green, N. Kondratenko, D. Steinberg, and O. Quehenberger. 1997. Characterization of CLA-1, a human homologue of rodent scavenger receptor BI, as a receptor for high density lipoprotein and apoptotic thymocytes. *J. Biol. Chem.* **272:** 17551–17557.
40. Xu, S., M. Laccotripe, X. Huang, A. Rigotti, V. I. Zannis, and M. Krieger. 1997. Apolipoproteins of HDL can directly mediate binding to the scavenger receptor SR-BI, an HDL receptor that mediates selective lipid uptake. *J. Lipid Res.* **38:** 1289–1298.
41. Gillotte, K. L., W. S. Davidson, S. Lund-Katz, G. H. Rothblat, and M. C. Phillips. 1998. Removal of cellular cholesterol by pre-β-HDL involves plasma membrane microsolubilization. *J. Lipid Res.* **39:** 1918–1928.
42. Galis, Z. S., G. K. Sukhova, R. Kranzhofer, S. Clark, and P. Libby. 1995. Macrophage foam cells from experimental atheroma constitutively produce matrix-degrading proteinases. *Proc. Natl. Acad. Sci. USA.* **92:** 402–406.
43. Vogel, R. A. 1997. Coronary risk factors, endothelial function, and atherosclerosis: a review. *Clin. Cardiol.* **20:** 426–432.
44. Gerrity, R. G., J. A. Goss, and L. Soby. 1985. Control of monocyte recruitment by chemotactic factor(s) in lesion-prone areas of swine aorta. *Arteriosclerosis.* **5:** 55–66.
45. Bath, P. M., A. M. Gladwin, and J. F. Martin. 1991. Human monocyte characteristics are altered in hypercholesterolaemia. *Atherosclerosis.* **90:** 175–181.
46. Weber, C., W. Erl, K. S. Weber, and P. C. Weber. 1997. HMG-CoA reductase inhibitors decrease CD11b expression and CD11b-dependent adhesion of monocytes to endothelium and reduce increased adhesiveness of monocytes isolated from patients with hypercholesterolemia. *J. Am. Coll. Cardiol.* **30:** 1212–1217.
47. Kimura, M., I. Kurose, J. Russell, and D. N. Granger. 1997. Effects of fluvastatin on leukocyte-endothelial cell adhesion in hypercholesterolemic rats. *Arterioscler. Thromb. Vasc. Biol.* **17:** 1521–1526.
48. Boring, L., J. Gosling, M. Cleary, and I. F. Charo. 1998. Decreased lesion formation in CCR2$^{-/-}$ mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature.* **394:** 894–897.
49. Gu, L., Y. Okada, S. K. Clinton, C. Gerard, G.K. Sukhova, P. Libby, and B. J. Rollins. 1998. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol. Cell.* **2:** 275–281.
50. Miller, N. E., A. La Ville, and D. Crook. 1985. Direct evidence that reverse cholesterol transport is mediated by high-density lipoprotein in rabbit. *Nature.* **314:** 109–111.
51. Ji, Y., B. Jian, N. Wang, Y. Sun, M. L. Moya, M. C. Phillips, G. H. Rothblat, J. B. Swaney, and A. R. Tall. 1997. Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux. *J. Biol. Chem.* **272:** 20982–20985.
52. Butcher, E. C. 1991. Leukocyte-endothelial cell recognition: Three (or more) steps to specificity and diversity. *Cell.* **67:** 1033–1036.
53. Cybulsky, M. I. and M. A. Gimbrone, Jr. 1991. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science.* **251:** 788–791.
54. Gerszten, R. E., Y. C. Lim, H. T. Ding, K. Snapp, G. Kansas, D. A. Dichek, C. Cabanas, F. Sanchez-Madrid, M. A. J. Gimbrone, A. Rosenzweig, and F. W. Luscinskas. 1998. Adhesion of monocytes to vascular cell adhesion molecule-1-transduced human endothelial cells: implications for atherogenesis. *Circ. Res.* **82:** 871–878.