HMG-CoA Reductase Inhibition Reduces Monocyte CC Chemokine Receptor 2 Expression and Monocyte Chemoattractant Protein-1–Mediated Monocyte Recruitment In Vivo

Ki Hoon Han, MD*; Jewon Ryu, MS*; Kyung Hee Hong, PhD; Jesang Ko, PhD; Youngmi Kim Pak, PhD; Jae-Bum Kim, PhD; Seong Wook Park, MD; Jae Joong Kim, MD

Background—The migration of circulating monocytes to the arterial wall during atherogenesis is largely modulated by activation of the CC chemokine receptor 2 (CCR2), a dominant monocyte chemotaxis receptor. The present study investigated whether 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibition affects CCR2 gene expression and CCR2-dependent monocyte recruitment.

Methods and Results—Competitive reverse transcription–polymerase chain reaction analysis and flow cytometry showed that simvastatin, an HMG-CoA reductase inhibitor, dose-dependently reduced monocyte CCR2 mRNA and protein expression. Treatment of 21 normocholesterolemic men with simvastatin (20 mg/d for 2 weeks) decreased CCR2 protein and mRNA expression in circulating monocytes. Promoter and electrophoretic mobility shift assays showed that simvastatin activated a peroxisome proliferator response element in THP-1 monocytes. Moreover, simvastatin-induced CCR2 downregulation was completely reversed by the synthetic peroxisome proliferator–activated receptor-γ antagonist GW9662. Simvastatin-treated monocytes showed little chemotaxis movement in response to monocyte chemoattractant protein-1 (MCP-1), a specific CCR2 ligand. Treatment of C57/BL6 mice with simvastatin (0.2 μg/g body weight IP, daily for 1 week) inhibited transmigration of CD80+ monocytes to the MCP-1–injected intraperitoneal space. Moreover, few circulating inflammatory cells from simvastatin-treated Sprague-Dawley rats (0.2 μg/g body weight IP, daily for 2 weeks) were recruited to the aortic wall of hypercholesterolemic littermates.

Conclusions—The inhibition of CCR2/MCP-1–dependent monocyte recruitment by simvastatin may prevent excessive accumulation of monocytes in the arterial wall during atherogenesis. (Circulation. 2005;111:1439-1447.)

Key Words: cells receptors statins atherosclerosis

Beginning at the earliest stage of atherogenesis, monocytes are recruited to the vascular wall, where they differentiate to macrophages and lipid-laden foam cells. Transmigration of monocytes through the vascular endothelial layer by chemotaxis is an important step in completing the vascular trafficking of monocytes. Inflammatory cytokines and oxidized LDL in atheroma induce the production of monocyte chemoattractant protein-1 (MCP-1) by smooth muscle cells, fibroblasts, and endothelial cells. MCP-1 triggers chemotaxis and transendothelial migration of monocytes to inflammatory lesions by interacting with the CC chemokine receptor 2 (CCR2) in monocytes. Mice deficient in MCP-1 or CCR2 display a severe reduction in monocyte adhesion and chemotaxis and develop fewer atheromatous plaques under hypercholesterolemic conditions, suggesting that the activation of monocyte CCR2 by MCP-1 is crucial in the development of atherosclerosis.

The antiinflammatory and antiatherogenic properties of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) on the arterial wall and monocytes/macrophages in plaque have been extensively studied. Statins have been shown to inhibit MCP-1 expression in the atherogenic arterial wall, together with the suppression of cytokine, oxygen radical, tissue factor, metalloproteinase-9, and plasminogen activator inhibitor-1 production. We previously have shown that the condition of monocytes, including CCR2 expression level, is as important as MCP-1 expression in the arterial wall for controlling the process of monocyte recruitment. In the present study, we therefore tested the hypothesis that statins may inhibit the transmigra-
tory activity of circulating monocytes. The present study demonstrates that HMG-CoA reductase inhibition by simvastatin reduces gene expression of monocyte CCR2 and MCP-1–mediated monocyte transmigration in vivo. This study further shows that the modulation of monocyte activities by simvastatin is sufficient to inhibit transmigration of monocytes to the arterial wall under hypercholesterolemic conditions in vivo. Moreover, we found that the decreased monocyte CCR2 expression by simvastatin involves peroxisome proliferator–activated receptor (PPAR)-γ–dependent intracellular signaling events.

Methods

Cell Culture and Maintenance

THP-1 monocytes maintained in RPMI 1640 containing 5% fetal bovine serum14,15 were incubated with simvastatin (provided by Merck & Co, Inc; 10 μmol/L), rosiglitazone (10 μmol/L), the specific PPAR-γ antagonist GW9662 (Alexis GmbH, 10 μmol/L), geranylgeranylated GP (2.5 μmol/L) or farnesylated GP (2.5 μmol/L); Sigma), free cholesterol (20 μg/mL), C3 transferase protein (2.5 μg/mL). The proportion of dead cells, as determined by flow cytometry after propidium iodide staining, was <5% in all experiments.

Transfection and Promoter Assay

The peroxisome proliferator response element (PPRE)–luciferase (Luc) pRL-TK vector (350 ng/μL distilled water) was mixed with 2 μL Magapectin (Qiogene Inc); 3 μL of 20 mmol/L HEPES, 1.9 μL distilled water, and 0.1 μL enhancer (Qiogene Inc); and incubated for 15 minutes at room temperature in the dark. This mixture was added dropwise to THP-1 monocytes (2 × 10× cells/mL), and a luciferase assay was performed 24 hours later with use of a -galactosidase vector–transfected monocytes. Results were expressed as light units and normalized relative to the amount of protein.

Quantification of CCR2 Transcripts

For quantitative assessment of CCR2 transcript levels, competitive reverse transcription–polymerase chain reaction (RT-PCR) analysis with the truncated CCR2 cDNA as a competitor was performed as previously described.16 A truncated form of CCR2 cDNA was developed by deleting the sequences between nucleotide 481 and nucleotide 666 of CCR2 cDNA and cloning the fragment into the pcDNA3.1 plasmid. With use of the primers 5'-ATGCTGTTCC-ACTTCTGGTCTCG-3' (sense) and 5'-TTATAAACCAGCG-AGAATCTGTCG-3' (antisense), full-length CCR2 cDNA (1083 bp) and the serially diluted competitor (898 bp) were coamplified for 30 cycles in the same reaction tube. After electrophoresis on 2% agarose gels, densities of the PCR product were measured with Un-Scan-It software and plotted; the point at which both curves intersected indicated equal concentrations of both. As an internal standard, human glyceraldehyde 3-phosphate dehydrogenase was amplified with the sense primer 5'-TCGGAGTCAACGATTGCTAG-3' and the antisense primer 5'-ATGGACTGTGTTGTCAGTCCTTTC-3' to ensure equal analysis conditions for competitive RT-PCR.

Analysis of Cell Surface CCR2

The amount of CCR2 protein on the monocyte cell surface was estimated by flow cytometry.16 In brief, 10× monocytes were incubated for 30 minutes at 4°C with 0.5 μg phycoerythrin (PE)-labeled mouse anti-CCR2 IgG (Pharmingen) in the presence of 5 μg unlabeled, nonspecific mouse IgG to saturate the nonspecific binding sites. Cell-associated fluorescence was measured and analyzed by fluorescence-activated cell sorting and Cell Quest software (FACS-can, Becton Dickinson). As a control, cells were incubated with PE-conjugated, nonspecific mouse IgG. CCR2 surface expression was expressed as specific mean fluorescence intensity (MFI), obtained by subtracting the MFI of control cells from that of cells labeled with anti-CCR2 antibody.

Determination of Cellular Cholesterol

Cellular lipids were extracted from 10× monocytes with chloroform/methanol (2:1 vol/vol), dried under N2, and resuspended in 50 mL isopropanol. Free and total cholesterol contents after hydrolysis of cholesterol ester with cholesterol hydrolase were estimated by the fluorometric method, and the amount of cholesterol ester was calculated by subtracting free cholesterol from total cholesterol.14

Electrophoretic Mobility Shift Assay

To analyze simvastatin-stimulated PPAR-γ activation, an electrophoretic mobility shift assay (EMSA) with nuclear extracts of simvastatin-treated THP-1 monocytes was performed as described.14 The oligonucleotides containing sequences of PPAR binding site 5'-CAAACACTGGTCAAGGTC-3' (Santa Cruz Biotechnology Inc) were end-labeled with [γ-32P]ATP (10 μCi at 110 TBq/nmol, Amersham Life Science) by T4 polynucleotide kinase, reacted for 20 minutes at room temperature with 5 μg nuclear proteins, and run on nondenaturing 6% polyacrylamide gels by electrophoresis for 2 hours. The gels were dried and exposed to x-ray films. To ensure that the developed bands were specific, a cold reaction was simultaneously performed with a 25-fold excess of unlabeled oligonucleotides. Moreover, we confirmed that the bands had shifted upward in the presence of 4 μg PPAR-γ2 antibody during the reaction (Alexis).

Chemotaxis Assay

The chemotactic activity of THP-1 monocytes in response to 2 nmol/L MCP-1 was measured in a 48-well microchemotaxis Boyden chamber (Neuroprobe) as described elsewhere.14 Monocytes that had transmigrated through the micropore and that were still bound to the polycarbonate membrane (5-μm pore size, Poretics) were fixed with 4% PFA, washed, and stained with 0.01% crystal violet. Cells on the polycarbonate membrane were counted in 4 random, high-power fields (×400). The result was expressed as the number of cells migrating in response to MCP-1 or to chemotaxis buffer.

Estimation of Monocyte Recruitment to the Peritoneal Cavity

Four-week-old, male C57BL/6 mice were treated with simvastatin as described.18 In brief, simvastatin in 100% ethanol (10 mg/mL) was...
diluted with 0.9% NaCl (final concentration, 10 μg/mL) and injected once into the intraperitoneal space (0.2 μg/g body weight [BW]). After 1 week, JE/MCP-1 (200 ng) was injected into the peritoneal cavity of each mouse. The mice were euthanized after 48 hours, and 10 mL PBS containing 10^4 PE-conjugated microbeads (Becton Dickinson) was injected into the peritoneal cavity of each mouse. Cells and beads in the suspension were withdrawn after 2 to 3 minutes and were pelleted by centrifugation (500 g, 5 minutes, 4°C).

To label the monocytes/macrophages, each cell preparation was incubated with 0.5 μg PE-conjugated anti-CD80 antibody in the presence of 5 μg unlabeled, nonspecific mouse IgG. The number of CD80^+ cells per 10^3 PE-conjugated microbeads was counted by FACScan, and the total number of CD80^+ cells in each peritoneal cavity was calculated. The protocol was reviewed and approved by the Animal Subjects Committee of the Asan Medical Center (Seoul, South Korea).

Estimation of Monocyte Recruitment to the Vessel Wall
Five-week-old, male Sprague-Dawley rats were fed a chow diet for 2 weeks, and simvastatin was injected once into the peritoneal space (0.2 μg/g BW) as described earlier. In contrast, 5-week-old female littermates were fed a high-cholesterol diet (1% cholesterol and 0.5% cholic acid) for 4 weeks to activate the arterial wall. A pair of rats (1 male and 1 female) were anesthetized by injection of pentobarbital (60 mg/kg BW IP). The common carotid artery and internal jugular vein of each were cannulated with polyethylene PE50 tubes (Becton Dickinson), and each was connected to the other rat’s jugular vein and common carotid artery, respectively; blood circulation between the rats was maintained for 30 minutes without interruption.

To detect male inflammatory leukocytes that had been recruited to the female aortic wall, the Sry1 gene in the Y chromosome (GenBank No. AF274872) was amplified by PCR with use of the primers 5'-AAGCAGCTGGGATATCAGTG-3' (sense) and 5'-TACCTATCACAGGCTTTGC-3' (antisense). The first PCR step used 50 ng genomic DNA for 20 cycles. In the second PCR step, 2 μL of the preamplified PCR product (10% vol/vol) from the first step was amplified for 30 cycles under identical conditions. Each PCR cycle

Figure 1. Effect of simvastatin (SIMVA) on monocyte CCR2 expression. A, THP-1 monocytes were treated for 24 hours with simvastatin in presence or absence of mevalonate (MEVA), and CCR2 surface expression was determined by flow cytometry with PE-conjugated, anti-CCR2 mouse IgG and expressed as MFI. *P<0.05, **P<0.01 by Mann-Whitney U test. Histogram in graph represents CCR2 surface expression on THP-1 monocytes with (dashed line) or without (bold line) simvastatin treatment (10 μmol/L over 24 hours). PE-conjugated, nonspecific mouse IgG was used to measure nonspecific binding (fine line). B, THP-1 monocytes were incubated for 24 hours in absence (Control) or presence of 10 μmol/L simvastatin (SIMVA), and CCR2 mRNA amount was measured by competitive RT-PCR. Diluted competitor, truncated CCR2 cDNA (●), was coamplified with CCR2 transcripts (CCR2, ○), and density of PCR product on 2% agarose gels was measured. Point at which both curves intersect (arrows) indicates equal amounts of CCR2 transcripts and CCR2 competitor. C, THP-1 monocytes were treated for 24 hours with simvastatin (SIMVA) in presence or absence of isoprenoid byproducts GGPP or FPP, and CCR2 surface expression was determined by flow cytometry as described in legend to above. MFI values specific for CCR2 were expressed relative to that in untreated control THP-1 monocytes (100%). *P<0.05 by Mann-Whitney U test.
consisted of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute. PCR products were electrophoresed on 1.2% agarose gels, and the density of each band was analyzed by densitometric scanning (Un-Scan-It). The protocol was reviewed and approved by the Animal Subjects Committee of the Asan Medical Center (Seoul, South Korea).

Other Analytical Procedures
Serum cytokine concentrations were measured with commercial ELISA kits (R&D Systems). Plasma triglyceride and total and HDL cholesterol levels were analyzed in the Lipid Analytical Laboratory of the Asan Medical Center and were measured by enzymatic techniques on an Abbott VP analyzer (Abbott Laboratories) with cholesterol (Boehringer Mannheim) and triglyceride (Abbott Laboratories) reagents. Data are expressed as mean±SD and were analyzed by the nonparametric Mann-Whitney U test.

Results

Simvastatin Downregulates CCR2 Expression in THP-1 Monocytes
Our previous studies proved that CCR2 expression in THP-1 monocytes is comparable to that in human circulating monocytes and that LDL and HDL cholesterol and rosiglitazone (a PPAR-γ ligand) regulate CCR2 expression to the same extent on THP-1 and human circulating monocytes. Therefore, we used THP-1 monocytes to analyze the effect of simvastatin on monocyte CCR2 expression in vitro. Flow cytometry showed that simvastatin dose-dependently decreased CCR2 expression on the cell surface, with a maximal reduction of 50% within 24 hours (Figure 1A). Coincubation with mevalonate (250 μmol/L) completely reversed the downregulatory effect of simvastatin (10 μmol/L) on CCR2 expression and further increased CCR2 expression to a level above that observed in the absence of simvastatin.

To clarify whether simvastatin-induced CCR2 downregulation involves gene transcription, we performed competitive RT-PCR with a specific competitor, the truncated CCR2 cDNA. We found that treatment of THP-1 monocytes for 24 hours with 10 μmol/L simvastatin significantly decreased CCR2 mRNA by 50% (Figure 1B).

Simvastatin Downregulates Monocyte CCR2 Expression in Human Circulating Monocytes
To confirm that simvastatin downregulates monocyte CCR2 expression in vivo, 21 young, healthy, male, nonhypercholesterolemic volunteers were treated with 20 mg/d simvastatin for 2 weeks. The simvastatin medication did not significantly change their lipid profiles, including LDL cholesterol levels (Table 1). We found that simvastatin treatment reduced CCR2 protein and mRNA expression in circulating monocytes of these volunteers. Their serum MCP-1 levels also decreased significantly after simvastatin treatment, as described elsewhere (P<0.01, ANOVA). Two weeks after discontinuation of simvastatin treatment, monocyte CCR2 expression and serum MCP-1 levels returned to basal levels (P<0.01, ANOVA), indicating that these effects were specifically due to simvastatin treatment. On the other hand, the reduction in serum tumor necrosis factor-α concentrations observed after simvastatin treatment did not completely return to basal levels after the withdrawal period, and simvastatin had no effect on serum interleukin-6 levels (Table 1).

Table 1. Effect of Simvastatin on Serum MCP-1 Levels and CCR2 Expression in Circulating Monocytes

<table>
<thead>
<tr>
<th>Variable</th>
<th>Basal</th>
<th>Simvastatin</th>
<th>Washout</th>
<th>P</th>
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<tr>
<td>LDL, mg/dL</td>
<td>84.2±29.2</td>
<td>80.5±49.4</td>
<td>83.9±44.4</td>
<td>NS</td>
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<tr>
<td>HDL, mg/dL</td>
<td>49.3±7.4</td>
<td>50.5±9.2</td>
<td>53.3±9.6</td>
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<tr>
<td>CCR2 protein, MFI</td>
<td>19.2±6.4</td>
<td>13.5±4.5</td>
<td>22.8±7.2</td>
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<tr>
<td>CCR2 mRNA, % GAPDH</td>
<td>21.8±15.3</td>
<td>7.3±4.8</td>
<td>14.2±6.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>MCP-1, pg/mL</td>
<td>133.9±43.9</td>
<td>68.9±18.4</td>
<td>129.4±42.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TNF-α, pg/mL</td>
<td>12.6±5.9</td>
<td>8.2±4.3</td>
<td>9.4±3.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>12.5±1.7</td>
<td>11.6±1.4</td>
<td>12.4±2.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

TNF indicates tumor necrosis factor; IL, interleukin.

Twenty-one normocholesterolemic, healthy, male volunteers were treated with 20 mg/d simvastatin for 2 weeks and then discontinued for 2 weeks. Before (basal) and after (simvastatin) treatment and after the withdrawal period (washout), CCR2 expression in circulating monocytes and serum levels of lipids and cytokines were measured. Values are mean±SD, and statistical analysis was done by ANOVA.

![Figure 2](image-url) Changes in cellular cholesterol content induced by simvastatin and mevalonate in THP-1 monocytes. THP-1 monocytes were treated for 24 hours with 10 μmol/L simvastatin (SIMVA) in presence or absence of 250 μmol/L mevalonate (MEVA) and harvested, and free (FC) and esterified (CE) cell-associated cholesterol contents were measured by fluorometry, as described in Methods, and adjusted by cell number. *P<0.05, **P<0.01 by Mann-Whitney U test.
GGPP, not FPP, Partially Reverses the Downregulatory Effect of Simvastatin on Monocyte CCR2 Expression

Byproducts of the HMG-CoA reductase–dependent cholesterol synthesis pathway, including GGPP and FPP, are biologically active metabolites. To determine whether simvastatin-induced depletion of GGPP and FPP was involved in CCR2 downregulation, THP-1 monocytes were incubated for 24 hours with 10 μmol/L simvastatin in the presence of GGPP or FPP, and cell surface CCR2 expression was measured by flow cytometry. GGPP, but not FPP, partially reversed the simvastatin-induced downregulation of CCR2 expression (Figure 1C).

Simvastatin Reduces Cellular Cholesterol Content

We previously showed that cellular cholesterol content was positively associated with monocyte CCR2 expression.\textsuperscript{14,15} To determine whether simvastatin reduces cellular cholesterol content in monocytes without cholesterol loading, THP-1 monocytes were incubated for 24 hours with 10 μmol/L simvastatin under lipoprotein-depleting conditions (RPMI 1640 medium containing 5% human lipoprotein–depleting serum), and cellular cholesterol content was measured. Simvastatin significantly reduced cellular cholesterol contents by 30% (Figure 2). The addition of mevalonate (250 μmol/L) not only inhibited simvastatin-induced depletion of cellular cholesterol but also increased cellular cholesterol to a level 3- to 4-fold higher than that of untreated control cells.

Downregulatory Effects of Simvastatin on CCR2 Expression Are Mediated by PPAR-γ Activation

To determine whether simvastatin-induced CCR2 downregulation is mediated by signaling pathways involving PPAR-γ...
Simvastatin Inhibits the Accumulation of Monocytes/Macrophages in MCP-1–Injected Mouse Peritoneal Cavities

To determine the role of simvastatin in the regulation of MCP-1–mediated monocyte recruitment in vivo, we assayed its effect on the number of monocytes/macrophages accumulated in mouse peritoneal cavities in response to injection with MCP-1. Mice were treated for 1 week with simvastatin (0.2 μg/g BW IP). Mouse MCP-1 (JE/MCP-1, 200 ng) was injected into the peritoneal cavity, and after 48 hours, intraperitoneal cells were harvested and the number of CD80+ monocytes/macrophages was counted by flow cytometry (Figure 5A). Injection of JE/MCP-1 into untreated mice significantly increased the number of intraperitoneal CD80+ cells. Treatment with simvastatin profoundly inhibited JE/MCP-1–mediated transmigration and accumulation of CD80+ cells (Figure 5B).

Simvastatin Inhibits Monocyte Recruitment to the Arterial Wall

Male Sprague-Dawley rats were treated with simvastatin (0.2 μg/g BW IP) for 2 weeks. As expected, treatment with simvastatin reduced CCR2 mRNA expression in circulating monocytes by ≈50%, as determined by RT-PCR (data not shown), but did not change lipid profiles except for a mild reduction of serum triglyceride levels (Table 2). On the other hand, feeding of a high-cholesterol diet to female rats resulted in a 4-fold elevation of serum total cholesterol levels, as well as an elevation in serum triglyceride level and a modest reduction in HDL cholesterol (Table 2). RT-PCR analysis confirmed that MCP-1, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 transcripts were expressed in the aortic walls isolated from hypercholesterolemic female rats (data not shown). The connection of blood circulation as described in Methods introduced male circulating inflammatory cells into the aortic wall of hypercholesterolemic female rats. PCR analysis confirmed that the aortic walls harvested from hypercholesterolemic female rats showed Y chromosome–linked Sryl gene signals, indicating that inflammatory cells were being continuously recruited into the aortic walls under hypercholesterolemic conditions. On the other hand, little Sryl gene signal in the activated aortic walls was detected in parallel experiments with simvastatin-treated male rats (Figure 6).

Discussion

Transmigration of monocytes through endothelial monolayers is induced by chemotaxis, which is an essential step to complete monocyte recruitment to the vascular wall. The present study demonstrates novel evidence that simvastatin modulates MCP-1–mediated monocyte chemotactic activity. We found that simvastatin downregulated a dominant monocyte chemokine receptor, CCR2, in vitro and in vivo. In the present study, the amount of CCR2 cell surface protein mass in simvastatin-treated monocytes was estimated as 50% of that in control cells. In vivo data from 21 healthy, normocholesterolemic volunteers also showed a similar degree of CCR2 reduction after medication with simvastatin (20 mg/d). Because mice with a CCR2± genetic background show...
slower progression of atherosclerosis under hypercholesterolemic conditions,7 the negative regulatory effect of simvastatin on monocyte CCR2 expression may be potent enough to retard the process of atheromatous plaque formation.

The present study identifies simvastatin as a potential PPAR-γ activator in monocytes. We found that simvastatin reduced cholesterol contents in monocytes by 30% under lipoprotein-depleting conditions. Moreover, EMSA showed that simvastatin-induced PPRE activation was completely inhibited by free cholesterol. Therefore, the decrease in cellular cholesterol by simvastatin may activate PPAR-γ, which in turn downregulates monocyte CCR2 expression as described earlier.16 This hypothesis is strongly supported by our previous observation that cholesterol depletion leads to the production of endogenous PPAR-γ ligand by triggering the cleavage of sterol regulatory element binding proteins (SREBPs) and adipocyte determination differentiation dependent factor (ADD)-1/SREBP-1.19 Our in vivo experiments with normocholesterolemic volunteers showed that simvastatin-induced CCR2 downregulation was not due to a reduction of serum LDL cholesterol levels. Thus, the effect of simvastatin is primarily due to direct HMG-CoA reductase inhibition in circulating monocytes. In addition to cellular cholesterol depletion, the decreased amount of GGPP, a
nonsteroidal isoprenoid byproduct of the de novo cholesterol synthesis pathway, and subsequent RhoA inactivation may increase PPAR-γ activities. EMSA clearly showed direct PPRE activation by inhibition of Rho, especially RhoA. A previous study described similar results: inhibition of geranylgeranylation of RhoA and RhoB induced PPAR-α activation in a human HepG2 hepatoma cell line.22

We further proved that simvastatin-induced PPAR-γ activation is potent enough to modulate monocyte activities, such as the impairment of MCP-1–mediated chemotaxis. As we observed in our previous studies that showed HDL- and oxidized LDL–induced CCR2 downregulation,15,16 simvastatin, which reduces monocyte CCR2 surface expression by 50%, almost completely abolished monocyte transmigration in response to MCP-1. Because monocyte CCR2 activation not only triggers monocyte chemotaxis23 but also induces actin polymerization24 and integrin-dependent firm adhesion through membrane-bound β-integrin activation,25,26 its inhibition may synergistically retard the process of MCP-1–mediated monocyte transmigration.

Our in vivo animal experiments further confirmed that simvastatin-induced CCR2 downregulation in circulating monocytes is functionally relevant. We used a very low dose of simvastatin (0.2 μg/g BW IP per day) to treat rodents as described to ensure that our observations were not affected by an inappropriately high simvastatin dose. The present study clearly reproduced the in vitro findings that the treatment of mice with simvastatin inhibited monocyte transmigration and accumulation into MCP-1–injected peritoneal cavities. Shimizu et al27 previously showed similar results with cerivastatin, which reduced macrophage accumulation in allografted arteries in mice. Both in vivo experiments clearly demonstrate the inhibition of monocyte recruitment under inflammatory conditions by statins; however, those findings may also be attributed to the effect of statins on the arterial wall, ie, suppression of MCP-1 expression.27 Thus, in another in vivo experiment with Sprague–Dawley rats, we eliminated the effect of simvastatin on the arterial wall by directly introducing inflammatory cells from a simvastatin–treated rat into the blood circulation of a hypercholesterolemic littermate. Although the development of hypercholesterolemia had obviously activated the arterial wall, few inflammatory cells (including monocytes) from the simvastatin–treated rat had migrated to the activated arterial wall of the hypercholesterolemic rat. Therefore, our in vivo findings strongly suggest that the functional remodeling of monocytes with simvastatin, ie, CCR2 downregulation, may be enough to inhibit vascular trafficking of monocytes under hypercholesterolemic conditions.

In summary, the present study demonstrates novel pleiotropic effects of simvastatin, ie, monocyte CCR2 reduction and the impairment of CCR2/MCP-1–mediated monocyte recruitment in vivo. Depletion of the nonsteroidal isoprenoid compound GGPP and cellular cholesterol resulted in PPAR-γ activation and subsequent CCR2 downregulation. Simvastatin–induced CCR2 downregulation in circulating monocytes decreases monocyte recruitment to the arterial wall and may be sufficient to inhibit the development of atherosclerotic plaques.

### Table 2. Lipid Profiles of Sprague-Dawley Rats Fed an HCD or Treated With Simvastatin

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC, mg/dL</td>
<td>Control (n=7) 70.0±9.4</td>
<td>Simvastatin (n=7) 64.8±7.7</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>99.7±11.5</td>
<td>412.9±72.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TG, mg/dL</td>
<td>Control (n=6) 46.0±8.7</td>
<td>Simvastatin (n=8) 35.7±6.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>36.2±5.7</td>
<td>86.7±16.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>HDL, mg/dL</td>
<td>Control (n=6) 47.1±4.2</td>
<td>Simvastatin (n=6) 42.2±3.4</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>56.2±11.4</td>
<td>14.6±3.0</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

TC indicates total cholesterol; TG, triglycerides.

Male Sprague–Dawley rats were fed a normal chow diet with (simvastatin) or without (control) simvastatin treatment (0.2 μg/g BW daily) for 2 weeks. Female littermates were fed either normal chow (control) or an HCD for 2 weeks. Serum lipoprotein levels were measured after 2 weeks. Values are mean±SD, and statistical analysis was done by the Mann-Whitney U test.

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Figure 6. Simvastatin reduces recruitment of inflammatory cells to arterial wall. Male Sprague–Dawley rats were fed normal chow, with (SIMVA) or without (cont) simvastatin intraperitoneal injection (0.2 μg/g BW daily) for 2 weeks. Female littermates were fed normal chow (cont) or high–cholesterol diet (HCD) for 2 weeks. Blood circulation between pair of rats (1 male and 1 female) was connected as described in Methods and maintained for 30 minutes. Female aorta was harvested, and genomic DNA was purified. Number of male inflammatory cells recruited to hypercholesterolemic female arterial wall was semiquantified by PCR amplification of Y chromosome–specific Y chromosome gene. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified under identical conditions and used as internal standard.
monocytes was functionally relevant. Monocytes treated with simvastatin showed little MCP-1–mediated monocyte chemotaxis and transmigration and little recruitment to the activated vascular wall in vivo. This novel pleiotropic effect of simvastatin on circulating monocytes may prevent the excessive accumulation of monocytes on the arterial wall during the process of atherosclerosis.

Acknowledgments
This study was supported by grant No. PPR02A624110 from the Korean Ministry of Science and the medical school grants program of Merck & Co, Inc. J. Kim and K.H. Han were supported in part by grants 2002-282 and 2005-288 from the Asan Institute for Life Sciences.

References
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Circulation. 2005;111:1439-1447
doi: 10.1161/01.CIR.0000158484.18024.1F
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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