

Disruption of Stromal-Derived Factor-1/Chemokine Receptor 4 by Simvastatin

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Background: The alpha chemokine, stromal-derived factor (SDF)-1 is produced by bone marrow stromal cells and other cells, especially damaged tissues. SDF-1 receptor, a chemokine receptor 4 (CXCR4), is expressed on inflammatory cells and that SDF-1/CXCR4 axis plays a critical role in migration of inflammatory cells. In cardiovascular diseases, SDF-1 is produced by endothelial cells and plaques and that SDF-1 chemoattracts monocytes to the endothelial cells resulting in a local inflammation. Simvastatin, a cholesterol-lowering agent, is a general drug for treatment of cardiovascular diseases. However, its molecular mechanism has not yet been completely elucidated.

Method: Herein, we investigated the role of simvastatin on the SDF-1/CXCR4 axis by employing flow cytometry, RT-PCR, chemotaxis and adhesion assays.

Results: Simvastatin (i) downregulates CXCR4 expression on monocytic cell line (THP-1) and primary monocyte in a dose-dependent manner, (ii) inhibits adhesion of monocytes to endothelial cells and (iii) decreases SDF-1 production by endothelial cells. Moreover, preincubation with simvastatin significantly decreased the migration of THP-1 towards the SDF-1 gradient.

Conclusion: All together our data indicate that simvastatin inhibits the binding of monocytes to endothelial cells through disrupting of the SDF-1/CXCR4 axis.

Keyword: SDF-1, CXCR4, Monocytes, Atherosclerosis, Simvastatin

Introduction

Atherosclerosis is an inflammatory disease characterized by vascular lesions containing cholesterol, infiltrated immune cells and connective tissues.¹⁻³ Injury of endothelium leads to activation of chemokines and adhesion molecules which in turn results in monocytes and T cells recruitment into subendothelial space.^{4,5} In addition, monocyte recruitment and interaction with endothelial cells are crucial for many inflammatory processes including the initiation and progression of atherosclerotic lesions.⁶ Chemokines such as monocyte chemoattractant protein-1 (MCP-1), interleukin 8 (IL-8), and fractalkine (CX3CL1) are shown to play an important role in pathophysiology of atherogenesis by activating and directing

leukocytes into the atherosclerotic lesion.⁷⁻⁹ However, the potential functions of other chemokines in monocyte-endothelial interaction have not yet been fully studied. The stromal-derived factor-1 (SDF-1), an alpha chemokine which is produced by bone marrow stromal cells and other tissues (especially damaged ones), plays a critical role in migration of inflammatory cells including monocytes to the inflammation sites. SDF-1 receptor, CXCR4 is expressed on a variety of cells including inflammatory cells and the interaction between SDF-1 and CXCR4 plays a vital role in trafficking of inflammatory cells.^{10,11} SDF-1 has been shown to be expressed in smooth muscle cells (SMC), endothelial cells and macrophages in human atherosclerotic plaques, but not in normal vessels¹² and mediates adhesion of monocytes to endothelial cells.¹³ However, its molecular mechanism is not well understood.

Statins are orally administered inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-

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CoA) reductase, an enzyme that catalyzes the conversion of HMG-CoA to mevalonate, an early and ratelimiting step in cholesterol biosynthesis.¹⁴ Statins are effective cholesterol-lowering agents, which have been extensively used for primary and secondary prevention of cardiovascular disease and diabetes.¹⁵⁻¹⁷ Although the benefits of statins are primarily attributed to their lipid-lowering effects, accumulating evidence suggests the possibility of other beneficial effects independent from serum cholesterol levels.¹⁸ For example, statins have been found to reduce neointimal thickening in normocholesterolemic rabbits¹⁹, inhibition of matrix metalloproteinase 9 secretion and MCP-1-mediated migration of the monocytic cell line, THP-1.²⁰ In addition, recent publications have shown that atorvastatin reduces Gro- α and CXCL16 expression in endothelial and peripheral blood mononuclear cells of patients with coronary artery disease.^{21,22} Although SDF-1/axis is a key player in monocyte trafficking and adhesion to endothelial cells but the effect of statins in this axis has not yet been explored. Herein, we show that simvastatin may reduce monocyte-endothelial interaction by disrupting SDF-1/CXCR4 axis.

Patients and Methods

Cells culture

The monocytic cell line, THP-1, was obtained from American Type Culture Collection (Rockville, Maryland, USA) and cultured in RPMI 1640, supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 U/ mL penicillin and 100 μ g/mL streptomycin at 95% relative humidity and 5% CO₂ at 37 °C as described [20]. For experiments, THP-1 cells were incubated with different doses of simvastatin in RPMI1640 containing 5% FSC for 24 h. Monocytes-enriched mononuclear cell (MNC) were separated using a two step procedure with single gradients in each step as reported.²³ First, light density mononuclear cells (MNC) were separated by centrifugation using a Ficoll-Hypaque gradient (density = 1.070 g/ml Amersham Biosciences, Uppsala, Sweden) and afterwards monocytes were enriched by centrifugation using a slight hyperosmolar Percoll gradient (density = 1.064 g/ml). Both gradients were centrifuged at 25-35°C, 400 g for 35 min. Enriched monocytes were co-cultured with Human umbilical vein endothelial cells (HUVEC) cells in the presence or absence of 10 μ M simvastatin in RPMI 1640, supplemented with 5% FCS, at 37 °C for 72h.

Endothelial cells

HUVEC were cultured on gelatin-coated flasks in media consisting of M199, 10 mM L-glutamine, 250 IU/mL penicillin-streptomycin, 20% FCS (all from Invitrogen, Carlsbad, CA), and endothelial cell growth supplement (Collaborative Biomedical, Bedford, MA).

Chemotaxis assay

Cell migration was determined by chemotaxis assay.²⁰ THP-1 cells were preincubated with either 10 mM simvastatin, 10 ng/ml AMD3100 for 24 h at 37°C and 1 × 10⁵ cells were loaded onto the upper chamber of transwells. Pre-warmed serum-free medium (IMDM 0.1% BSA) containing SDF-1 (200 ng/ml) was added to the lower chambers and incubated at 37°C for 3 hours. The migrated cells in the lower chamber were then counted by flow cytometry.

Fluorescence-Activated Cell Sorting Analysis (FACS)

THP-1 cells were incubated with different concentrations of simvastatin for 24h and then stained with either PE-labelled anti-CXCR4 or isotype antibody for 30 min on ice, then washed three times with cold PBC and analyzed by FACS. MNC were stimulated with simvastatin for 24 and then co-stained with FITC-labelled CD14 and PE-labelled CXCR4 for 30 min on ice and washed three times with cold PBS. Flow cytometry was performed using a FACS Calibur instrument (Becton Dickinson).

Adhesion assay

It has been shown previously that HMG-CoA reductase inhibitor prevents binding of monocytes to endothelial cells.²⁴ THP-1 cells were washed three times with PBS, preincubated with 2 μ M Calcein- AM for 20 min at 37°C, washed three times with PBS again and incubated with either 10 μ M simvastatin or 10 ng/ml AMDM3100 for 30 min at 37 °C. THP-1 cells were then co-cultured on the top of HUVEC monolayers for 20 min, non-adherent cells were removed by gentle rocking and adherent cells were observed and evaluated by fluorescent microscopy. In some experiments monocytes-enriched MNC were co-cultured in the absence or presence of 10 μ M simvastatin for three days, then non-adherence cells were removed as mentioned above. The adherent cells were fixed by 2% paraformaldehyde, washed with cold PBS and nucleus were stained with Dabi for 15 min at 4°C

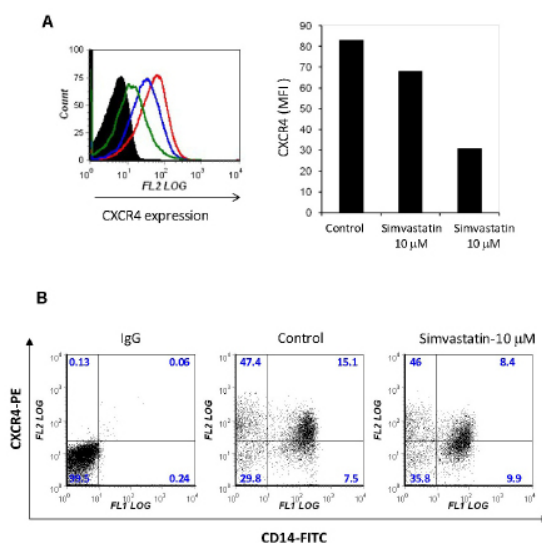


Figure 1. Simvastatin downregulates CXCR4 expression on monocytes. (A) THP-1 cells were incubated with different concentrations of simvastatin (SV) for 24h and the cells were then stained with either PE-labeled control mouse IgG or PE-labeled anti-CXCR4 MoAb and were analyzed by flow cytometry. Red, blue and green histograms show CXCR4 expression in control, 1mM and 10 mM simvastatin-treated THP-1 cells, respectively and black histogram shows staining for isotype IgG. (B) Normal monocyte-enriched MNC were incubated with 10 mM simvastatin for 24, co-stained with FITC-labeled anti-CD14 and PE-labeled anti- CXCR4 and were analyzed by flow cytometry. The data are representative of 3 independent experiments.

in the dark. Cells were visualised by fluorescent microscopy.

RT-PCR

RNA from HUVEC cells was extracted using TRIzol Reagent (Invitrogen Ltd, Paisley, UK) according to the manufacturer's instructions. To evaluate SDF-1 transcripts, RT-PCR reactions were carried out using primer sequences for human GAPDH (housekeeping gene), and SDF-1 as described previously.²⁵ Thermocycling was performed with an Eppendorf Mastercycler (Westbury, NY) and the PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide. Gels were visualized under UV light and photographed using the Xplorer D55 (UVItec Limited, Cambridge, UK). The relative level of target mRNA was regarded as the ratio between the intensities of the SDF-1 and the GAPDH bands. The t-test was performed on the densitometry data to determine statistical difference between the means of two sets of data.

Results

Simvastatin significantly downregulates CXCR4 expression on monocyte

As SDF-1/CXCR4 axis plays an important role in migration of inflammatory cells to the inflammation site, we examined whether simvastatin would have any effect on CXCR4 expression. Simvastatin significantly downregulates CXCR4 expression on monocytic cell line, THP-1, in a dose-dependent manner (Fig. 1A). Moreover, when we incubated primary monocytes with simvastatin, we found that simvastatin reduces CXCR4 expression on these cells as well (Fig. 1B).

Simvastatin reduces monocyte migration towards SDF-1

As simvastatin downregulates CXCR4, we examined whether this simvastatin-mediated CXCR4 downregulation would affect the migration of monocytes towards SDF-1. We have found that simvastatin significantly reduces chemotactic effects of SDF-1, indicating that simvastatin significantly reduces migration of monocytes towards SDF-1 (Fig. 2).

Simvastatin decreases binding of monocytes to HUVEC cells

Monocyte recruitment and interaction with endothelial cells have been shown to play a crucial

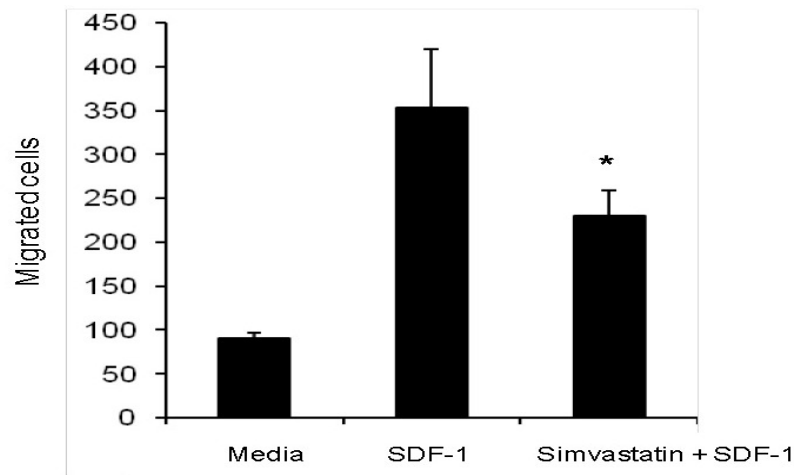


Figure 2. The effect of simvastatin on migration of THP-1 cells. Control or simvastatin(SV)-treated cells were loaded on the top of transwell and let them to migrate towards 200 ng/ml SDF-1 for 3 h. The migrated cells were counted by flow cytometry. * P value < 0.05 vs. SDF-1.

role for many inflammatory processes including the initiation and progression of atherosclerotic lesions.²⁶ Herein, we investigated whether simvastatin would affect the binding of monocytes to HUVEC cells and found that simvastatin significantly inhibits the binding of THP-1 cells to HUVEC cells (Fig 3A). As the specific inhibitor of CXCR4, AMD3100, has also reduced the adhesion of THP-1 cells to HUVEC cells, we conclude that binding of these cells to HUVEC cells are CXCR4-dependent. Importantly, when we co-cultured primary monocytes with HUVEC cells for three days, we observed a number of monocytes adhered to HUVEC cells. In contrast, simvastatin treatment remarkably reduced the number of adherent monocytes to HUVEC cells (Fig. 3 B and C).

The effect of simvastatin on SDF-1 expression in HUVEC cells

SDF-1 is produced by HUVEC, smooth muscle cells and atherosclerotic plaques and that SDF-1 chemoattracts monocytes and other inflammatory cells to the vasculatures.^{4,7} We have examined whether simvastatin would downregulates SDF-1 expression in HUVEC cells. Fig.4 shows that simvastatin reduces SDF-1 expression in HUVEC cells, indicating that simvastatin not only downregulates CXCR4 expression but also reduces SDF-1 expression in the local vasculature.

Discussion

Leukocyte–endothelial cell interaction is known as one of the pivotal mechanisms in the development of atherosclerosis, with a various family of bioactive molecules, such as adhesion molecules, chemokines, and their receptors, involved in that process.¹³ Statins have been found to decrease neointimal thickening in normocholesterolemic rabbits¹⁹ and reduces THP-1 migration by downregulation of matrix metalloproteinase (MMP)-9.²⁰ SDF-1 is a chemotactic factor for lymphocytes and monocytes and has also been detected histologically in rupture prone atherosclerotic plaque, indicating to play a role in atherosclerosis.¹² In addition, statins have been extensively used for primary and secondary prevention of cardiovascular disease, but their effects on SDF-1/CXCR4 axis has not yet been explored. In this study we showed that simvastatin downregulates CXCR4 on monocytes resulting in decreasing migration of monocytes towards SDF-1 gradients and reducing monocyte-endothelial adhesion. CXCR4 has been shown to be expressed on inflammatory as well as tumor cells and the interaction of SDF-1 with CXCR4 plays an important role in migration of monocytes and lymphocytes to the inflammation site as well as metastasis of tumor to the distance organs.^{27,28} Several reports have recently demonstrated that AMD3100, the specific antagonist of CXCR4, could results in reducing of inflammation and tumor

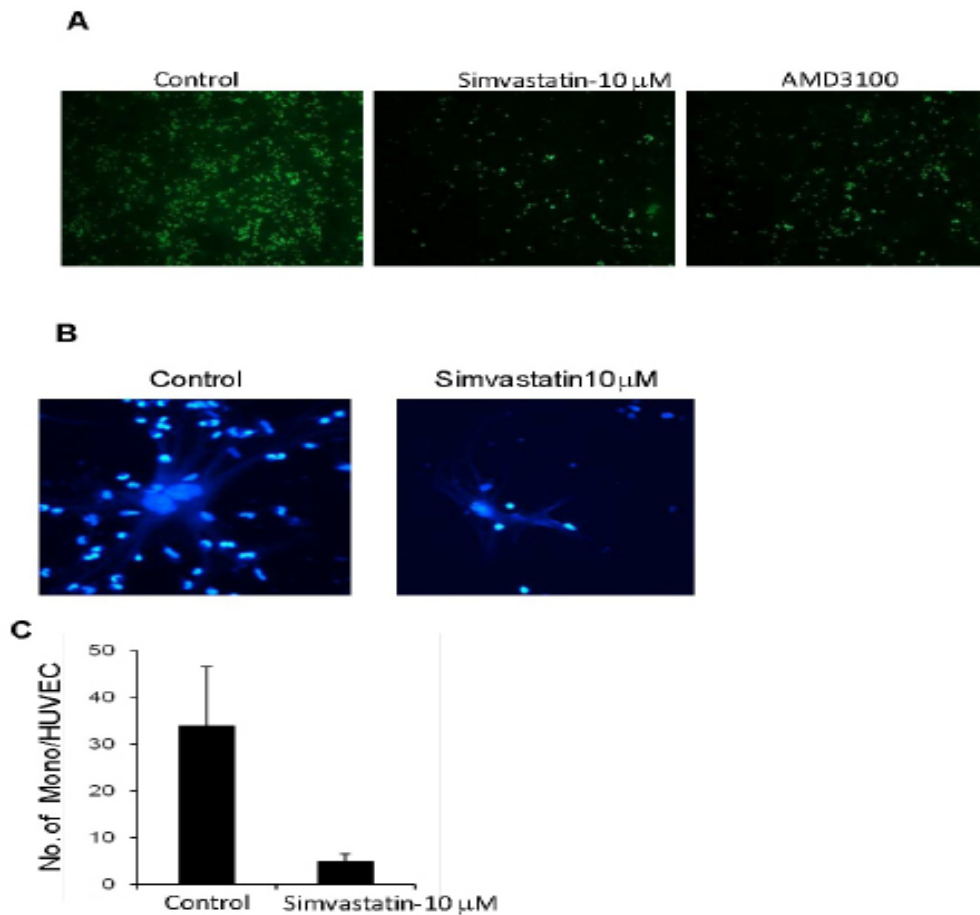


Figure 3. Simvastatin remarkably reduces the binding of THP-1 cells to HUVEC cells. (A) A microscopic data showing the effect of simvastatin (SV) on binding of THP-1 to HUVEC cells. THP-1 cells were labeled with Calcein for 30 min at 37°C, washed three times and preincubated with 10 μM simvastatin for 30 min then were layered on HUVEC cells and incubated at 37 for 20 min. Unattached THP-1 cells were removed by gentle washing and attached THP-1 cells were visualized by fluorescent microscope. (B) Shows binding of monocytes to HUVEC cells. Monocytes-enriched MNC were co-cultured with HUVEC cells in the absence or presence of 10 M SV for three days. None adherent cells were removed by gentle washing and adherent cells were stained with Dapi and developed by fluorescent microscopy. (C) Shows the No. of monocytes to each HUVEC cells.

metastasis.²⁹ Hyperlipidemia and lipid oxidation in endothelium layer of the vascular wall is a critical step resulting in formation of foam cells via uptake of oxidized (OX)-LDL by macrophages.³⁰ In addition, Guta SK, et al have recently shown that CXCR4 is upregulated in monocyte-derived macrophage and OX-LDL increase CXCR4 expression in these cells³¹, indicating an important role for SDF-1/CXCR4 axis in the pathobiology of vascular disease. In the current study we show for the first time that simvastatin significantly downregulates CXCR4 on primary monocytes and monocytic cell line THP-1 and those simvastatin-treated cells showed a

lower ability to migrate towards SDF-1 compared to untreated cells. In supporting of our data, a recent study showed that simvastatin suppresses THP-1 migration towards MCP-1.²⁰ Collectively, these data indicate that simvastatin, in addition to its cholesterol-lowering effect, may have a strong potential to act as anti-inflammatory drug.

Monocyte–endothelial contact through adhesion molecules can be enhanced by chemokines such as MCP-1, IL-8 and Gro- α which can induce arrest of cells from flow.³² In addition, a recent report has shown that SDF-1 enhances human monocyte cell adhesion to HUVECs, which was reduced by

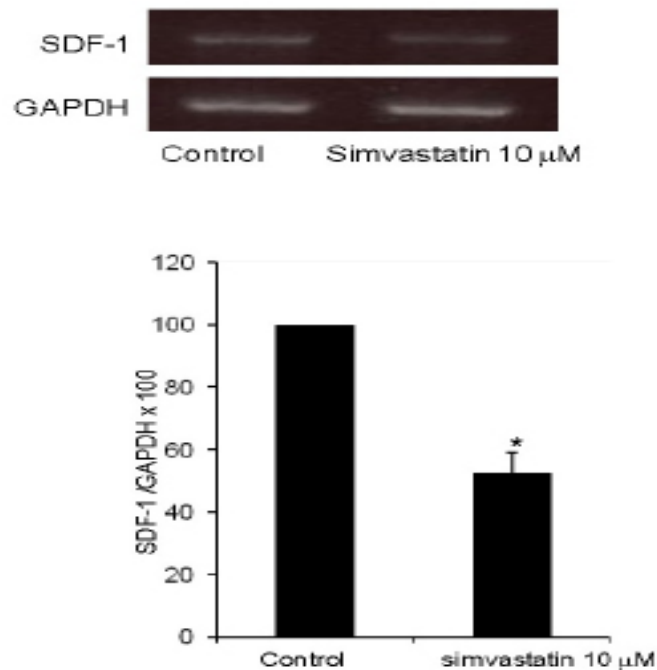


Figure 4. Simvastatin downregulates SDF-1 expression by HUVEC cells. (A) HUVEC cells were culture in IMDM in the absence or presence of 10 mM simvasatin for 24 and the SDF-1 expression was examined by RT-PCR. (B) Graph of densitometry of bands from 3 independent experiments. * P value < 0.05 vs. control.

azelnidipine treatment of monocytes via inhibition of PKC α .¹³ Consistently, here we show that simvastatin significantly reduces adhesion of primary monocytes and monocytic cell line, THP-1, to HUVEC cells. As the specific inhibitor of CXCR4, AMD3100, significantly reduces binding of these cells to HUVEC, we could concludes that simvastatin decreases binding of monocytes to HUVEC cells, at least partially by downregulation of CXCR4.

Detection of SDF-1 in atherosclerosis plaques¹² suggests that SDF-1 may play a critical role in attracting of inflammatory cells particularly monocytes to the atherosclerotic lesions. Moreover, previous studies have demonstrated that other chemokines such as Gro- α CXCL16, IL-8 and MCP-1 are also involved in pathophysiology of atherosclerosis.^{21,22,32,33} Aukrust HP, et al have elegantly shown that CXCL16 and Gro- α are elevated in serum of coronary artery disease compared to healthy controls and atorvastatin significantly reduced expression of these chemokines in endothelial cells and macrophages.^{21,22} Consistently, we show here that simvastatin downregulates SDF-1 in the

endothelial cells. All together, these data imply that at least, some of the therapeutic effects of statins in atherosclerosis patients are mediated by downregulation of chemokines in endothelial cells. On the other hand, it is a well known notion that SDF-1/CXCR axis is one of the key players in mobilization/homing of hematopoietic stem cell (HSC) and endothelial progenitors.^{34,35} Recent studies have already shown that downregulation of SDF-1 by mobilizing agent, granulocyte-colony stimulating factor (G-CSF) leads to mobilization of HSC into the circulation. However, the effect of simvastatin on HSC mobilization needs to be explored.

In conclusion, we found that simvastatin disrupts SDF-1/CXCR4 axis resulting in inhibiting binding of monocytes to endothelial cells, implying that the SDF-1/CXCR4 axis could be a potential target for the treatment of atherosclerosis. However, further in vivo studies are required.

The authors declare that they have no Conflicts of Interest.

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