Research Paper

Statins contribute to enhancement of the number and the function of endothelial progenitor cells from peripheral blood

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Abstract: The aim of the present study was to investigate whether fluvastatin augments the number of endothelial progenitor cells (EPCs), and promotes EPCs proliferation, migration and adhesion. Total mononuclear cells (MNCs) were isolated from peripheral blood by Ficoll density gradient centrifugation. The cells were then plated on fibronectin-coated culture dishes. After being cultured for 7 d, the attached cells were stimulated with fluvastatin (final concentrations: 0.01, 0.1, 1, 10 µmol/L), simvastatin (1 µmol/L) or a vehicle for the respective time points (6, 12, 24 and 48 h). EPCs were characterized as adherent cells double positive for DiLDL-uptake and lectin binding by direct fluorescent staining under a laser scanning confocal microscope. EPCs were further documented by demonstrating the expression of KDR, VEGFR-2 and AC133 with flow cytometry. EPCs proliferation, migration and in vitro vasculogenesis activity were assayed by MTT assay, modified Boyden chamber assay and in vitro vasculogenesis kit, respectively. EPCs adhesion assay was performed by replating it on fibronectin-coated dishes, and the adherent cells were then counted. In addition, we also studied EPCs culture assay of peripheral blood from fluvastatin-treated animals in vivo. Incubation of isolated human MNCs with fluvastatin dose- and time-dependently increased the number of EPCs, while reached the maximum 24 h after the administration at 1 µmol/L, (2.5-fold increase, P<0.05). Moreover, treatment of rats with fluvastatins elevated the number of EPCs (3-fold increase, P<0.05), thus extending the in vitro data. In addition, fluvastatin also promoted EPC proliferation, migration, adhesion and in vitro vasculogenesis in a concentration-dependent manner. The effects of fluvastatin on EPCs were compared with those of simvastatin at the same concentration (1 µmol/L), with a result of no statistical difference. The results of the present study define a novel mechanism of the action of statins: the augmentation of EPCs with enhanced functional activity.

Key words: statins; endothelial progenitor cells; coronary artery disease; vasculogenesis; rat

他汀类药物对外周血内皮祖细胞的影响

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摘 要: 本文旨在探讨他汀类药物氟伐他汀对外周血内皮祖细胞(endothelial progenitor cells, EPCs)数量和功能的影响。用密度梯度离心从外周血获取单个核细胞, 将其接种在人纤维连接蛋白(human fibronectin)包被的培养板中, 培养 7 d 后, 收集贴壁细胞, 加入不同浓度氟伐他汀(分别为 0.01、0.1、1、10 µmol/L)和辛伐他汀(1 µmol/L), 培养一定的时间(6、12、24、48 h), 用激光共聚焦显微镜鉴定 FITC-UEA-1 和 DiI-acLDL 双染色阳性细胞为正在分化的 EPCs, 用流式细胞仪检测其表面标志进一步鉴定 EPCs。在倒置荧光显微镜下计数。采用 MTT 比色法、改良的 Boyden 小室、粘附能力测定实验和体外血管生成试剂盒观察 EPCs 的增殖能力、迁移能力、粘附能力及体外血管生成能力。结果显示, 氟伐他汀显著增加外周血 EPCs 的数量, 并且 EPCs 数量随氟伐他汀浓度增加及作用时间延长而增加, 1 µmol/L 浓度氟伐他汀作用 24 h 对 EPCs 的数量影响最为显著(较对照组增加 2.5 倍, P<0.05)。在动物实验中, 喂养氟伐他汀 3 周后, 大鼠的 EPCs 也较对照组增加 2 倍(P<0.05), 进一步支持了体外实验的结果。氟伐他汀和辛伐他汀也显著改善了外周血 EPCs 的粘附能力、迁移能力、增殖能力和体外血管生成的能力, 相同浓度的氟伐他汀和辛伐他汀(1 µmol/L)对 EPCs 数量和功能的影响并无显著差异。上述观察结果提示他汀类药物可增加 EPCs 的数量, 改善 EPCs 功能。

关键词: 他汀类药物; 内皮祖细胞; 冠心病; 血管生成; 大鼠

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Studies have identified a cell population termed endothelial progenitor cells (EPCs) that have the capacity to circulate, proliferate, and differentiate into mature endothelial cells, but which have not yet acquired characteristic mature endothelial markers and have not yet formed a lumen \[1^{2-3}\]. Laboratory evidence has suggested that EPCs participate in postnatal neovascularization and reendothelialization \[1^{3-10}\]. Moreover, EPCs have recently been shown to affect the progression of coronary artery diseases (CAD) \[1^{11}\]. HMG-CoA reductase inhibitors (statins) have been developed as lipid-lowering drugs, and are well established to reduce morbidity and mortality from CAD \[1^{12,13}\]. In particular, statins have recently been reported to promote EPC proliferation, migration, and cell survival \textit{in vitro}\[1^{14-17}\].

Fluvastatin is a member of the HMG-CoA reductase inhibitor family of drugs that block the body’s production of cholesterol. The present study is to investigate whether fluvastatin can augment EPC numbers \textit{in vitro} and \textit{in vivo}, promote EPC proliferation, migration and adhesion, which might importantly contribute to the well-established beneficial effects of statins in patients with CAD.

1 MATERIALS AND METHODS

1.1 Isolation and cultivation of EPCs. EPCs were cultured according to previously described techniques \[10,18\]. Briefly, total mononuclear cells (MNCs) were isolated from peripheral blood of healthy human volunteers by Ficoll density gradient centrifugation. Cells were plated on culture dishes coated with human fibronectin (Chemicon) and maintained in Medium 199 (Sigma) supplemented with 20 percent fetal-calf serum, VEGF (10 ng/ml, Chemicon), penicillin (100 U/ml), and streptomycin (100 µg/ml). After 4 d in culture, nonadherent cells were removed by washing with PBS, new media was applied, and the culture was maintained through d 7. Before cytochemical analysis, cells were serum depleted for 24 h. Experiments were initiated by addition of the indicated amount of activated fluvastatin to make a series of final concentrations: 0.01, 0.1, 1, 10 µmol/L, simvastatin (1 µmol/L) or vehicle control for the respective time points (6, 12, 24 and 48 h).

1.2 Cellular staining. Fluorescent chemical detection of EPCs was performed on attached MNCs after 7 d in culture. Direct fluorescent staining was used to detect dual binding of FITC-labeled Ulex europaeus agglutinin (UEA-1; Sigma) and 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine (DiI)-labeled acetylated low density lipoprotein (acLDL; Molecular Probe). Cells were first incubated with acLDL at 37°C and later fixed with 2% paraformaldehyde for 10 min. After washed with PBS, the cells were reacted with UEA-1 (10 µg/ml) for 1 h. After the staining, samples were viewed with an inverted fluorescent microscope (Leica DMR) and further demonstrated by a laser scanning confocal microscope (LSCM, Leica). Cells demonstrating double-positive fluorescence were identified as differentiating EPCs \[1^{11,16-18}\]. Two or three independent investigators evaluated the number of EPCs per well by counting 15 randomly selected high-power fields with an inverted fluorescent microscope.

1.3 Flow cytometry analysis. Fluorescence-activated cell sorting (FACS) detection of EPCs was performed on attached MNCs after 7 days in culture. Mononuclear cells were detached with 0.25% trypsin followed by repeated gentle flushing through a pipette tip. Cells (2×10^6) were incubated for 30 min at 4°C with anti-vascular endothelium (VE)-cadherin (Chemicon), phycoerythrin-conjugated monoclonal antibodies against kinase insert domain-containing receptor (KDR, R&D), CD34, AC133 (Miltenyi Biotec). A FITC-conjugated anti-mouse antibody (Vector) was added for staining with VE-cadherin. Isotype-identical antibodies served as controls. After treatment, the cells were fixed in 1% paraformaldehyde. Quantitative FACS was performed on a FACStar flow cytometer (COULTER) \[1^{11,18}\].

1.4 Migration assay. EPC migration was evaluated by using a modified Boyden chamber (Qiling Medical Equipment Factory, Jiangsu, China) assay. In brief, Isolated EPCs were detached using 0.25% trypsin, harvested by centrifugation, resuspended in 500 µl M199, and counted, then 2×10^4 EPCs were placed in the upper chamber of a modified Boyden chamber. Fluvastatin, simvastatin or vehicle control, in serum-free M199 media, was placed in the lower compartment of the chamber. After 24 h incubation at 37°C, the lower side of the filter was washed with PBS and fixed with 2% paraformaldehyde. For quantification, cells were stained with Giemsa solution. Cells migrating into the lower chamber were counted manually in 3 random microscopic fields \[1^{11,14}\]. All groups were studied in triplicate.

1.5 Cells adhesion assay. After 24 h of incubation with fluvastatin, simvastatin or vehicle control, human EPCs were washed with PBS and gently detached with 0.25%
trypsin. After centrifugation and resuspension in M199, 5% FBS, identical cell numbers were replated onto fibrinogen-coated culture dishes and incubated for 30 min at 37°C. Adherent cells were counted by independent blinded investigators[9]. All groups were studied in triplicate.

1.6 EPCs proliferation assay. The effect of fluvastatin or simvastatin on EPCs proliferation was determined by MTT assay. After being cultured for 7 d, EPCs were digested with 0.25% trypsin and then cultured in serum-free medium in 96-well culture plate (200 µl per well), to which was added fluvastatin (to make a series of final concentrations: 0.01, 0.1, 1, 10 µmol/L), simvastatin (1 µmol/L) or vehicle control. Each concentration included six wells, while the serum-free medium served as a control. After being cultured for 24 h, EPCs were supplemented with 10 µl MTT (5 g/L) and incubated for another 6 h. Then the supernatant was discarded by aspiration and the EPC preparation was shaken with 200 µl DMSO for 10 min, before the OD value was measured at 490 nm. All groups were studied in triplicate.

1.7 In vitro vasculogenesis assay. In vitro vasculogenesis assay was performed with in vitro Angiogenesis Assay Kit (Chemicon). The protocol was according to the manufacturer’s instructions. Briefly, ECMatrix™ solution was thawed on ice overnight, then mixed with 10 × ECMatrix™ Diluent and placed in a 96-well tissue culture plate at 37°C for one hour to allow the matrix solution to solidify. EPCs were harvested as described above and replated (10000 cells per well) on top of the solidified matrix solution. Cells were grown with fluvastatin, simvastatin or vehicle control, and incubated at 37°C for 12 h. Tubule formation was inspected under an inverted light microscope at 200 × magnification. Tubule formation was defined as a structure exhibiting a length 4 times its width. Five independent fields were assessed for each well, and the average number of tubules/200 × field determined. All groups were studied in triplicate.

1.8 Animal experiments. Age-matched male Sprague-Dawley rats (laboratory animal center, medical college, zhejiang university) were fed with a daily oral dose of 20 mg/kg fluvastatin for 3 weeks (n=10 animals). Control mice (n=10 animals) were kept without fluvastatin. Both groups of rats were fasted overnight, and blood obtained by heart puncture was collected into serum tubes. Four days after culture, EPCs, recognized as attaching spindle-shaped cells, were assayed by costaining with acLDL-DiI and FITC-conjugated BS-1 lectin (Sigma). Independent investigators used LSCM to identify double positive cells as EPCs and manually count with inverted fluorescence microscopy.

1.9 Statistical analysis. All data are presented as mean±SD. Differences between group means were assessed by an unpaired Student’s t test for single comparisons. Values of P<0.05 were considered significant.

2 RESULTS

2.1 Characterization of human EPCs

Total MNCs isolated and cultured for 7 d resulted in a spindle-shaped, EC-like morphology (Fig. 1). EPCs were characterized as adherent cells double positive for DiLDL-uptake and lectin binding by using LSCM (Fig. 2). They were further documented by demonstrating the expression of VE-cadherin (76±8.6%), KDR (78±7.8%), CD34 (28.7±6.9%), and AC133 (17.1±8.1%) by flow cytometry. Other investigators have previously demonstrated that endothelial progenitor cells isolated in this fashion also exhibit many other endothelial characteristics, including expression of CD31, vWF, and vascular endothelial growth factor receptor 2 (VEGFR-2), and so on[11,18].

2.2 Statins increased EPCs in vitro

Incubation of isolated human MNCs with fluvastatin for 24 h increased the number of differentiated, adherent EPCs in a concentration-dependent manner, with a peak at 1 µmol/L (2.5-fold increase, Fig. 2 and 3). In time-course experiments performed with a fluvastatin concentration of 1 µmol/L, increase of EPCs number became apparent at 12 h and reached the maximum at 24 h (2.5-fold increase, Fig. 2 and 3). Similarly, incubation of isolated human MNCs with HMG-CoA reductase inhibitors simvastatin at 1 µmol/L for 24 h also augmented the number of EPCs up to 2.67 fold. However, there was no statistical difference between the effects of fluvastatin on EPCs number and those of simvastatin at the same concentration.

2.3 Effects of statins on EPC proliferation

The effects of fluvastatin on EPCs proliferation were assayed using a MTT assay (Fig. 4). Fluvastatin increased EPCs proliferative activity, maximal at 1 µmol/L fluvastatin (control vs 1 µmol/L fluvastatin: 0.52±0.106 vs 0.826 ±0.131; 490 nm light absorbance, P<0.05). However, there was no statistical difference between the effects of fluvastatin on EPC proliferation and those of simvastatin at the same concentration (0.833±0.141 vs 0.826±0.131, P>0.05).

2.4 Effects of statins on EPC migration
Fig. 1. A: Mononuclear cells were plated on culture dishes coated with human fibronectin just after being isolated from peripheral blood. B: After 7 d in culture, nonadherent cells were removed, and the attached cells appeared spindle-shaped and EC-like in morphology. Scale bar, 50 µm.

Fig. 2. Mononuclear cells were incubated with fluvastatin (1 µmol/L) and a vehicle as control for 24 h, and adherent cells DiLDL uptake (red, exciting wave-length 543 nm) and lectin binding (green, exciting wave-length 477 nm) were assessed with a laser scanning confocal microscope. Double positive cells appear yellow in the overlay. A1-A3, control group. B1-B3, fluvastatin group (1 µmol/L). Scale bar, 25 µm.
The effects of fluvastatin on EPCs migration were analyzed in a modified Boyden chamber assay (Fig. 5). Fluvastatin profoundly enhanced cell migration, maximal at 1 µmol/L fluvastatin (control vs 1 µmol/L fluvastatin, 5 ± 4 vs 42 ± 16, cells per high-powered field, \( P < 0.05 \)). Similarly, simvastatin at concentration of 1 µmol/L profoundly enhanced cell migration (control vs 1 µmol/L simvastatin, 5 ± 4 vs 45 ± 19, \( P < 0.05 \)). There was no statistical difference between the effects of fluvastatin on EPCs migration and those of simvastatin at the same concentration (42 ± 16 vs 45 ± 19, \( P > 0.05 \)).

2.5 Effects of statins on EPC adhesiveness

To study the possibility that fluvastatin alter adhesiveness of cultured human EPCs, EPCs were incubated with fluvastatin for 24 h. After replating on fibronectin-coated dishes, EPCs preexposed to fluvastatin exhibited a significant increase in the number of adhesiveness cells at 30 min (Fig. 6). The increase in the number of adhesiveness cells occurred dose-dependently with a maximal effect achieved at 1 µmol/L. Simvastatin at concentration of 1 µmol/L also significantly increase the number of adhesiveness cells. However, There was no statistical difference between the effects of fluvastatin on EPC adhesiveness and those of simvastatin at the same concentration. These findings suggest that statins modulate the adhesiveness of EPC to support homing to vascular injury.

2.6 Effects of statins on EPC vasculogenesis

Recent studies have demonstrated that circulating EPCs home to sites of neovascularization and differentiate into endothelial cells in situ\(^{[6]}\) in a manner consistent with a process termed vasculogenesis. In vitro vasculogenesis assay was to simulate this process, and here was used to investigate the ability of EPCs to participate in neovascularization, which is the most important activity of EPCs. The response of the EPCs to fluvastatin and simvastatin was depicted in Fig. 7. Tubule number increased in a dose-response to fluvastatin concentrations at 12 h of incubation, with peak production at 1 µmol/L fluvastatin. Moreover,
tubules in the fluvastatin wells were qualitatively different and more complex than those in the control wells. Similarly, simvastatin (1 µmol/L) also significantly increased tubule number.

2.7 Statins increased EPC levels \textit{in vivo}

To test the \textit{in vivo} relevance of our findings, rats were fed with fluvastatin (20 mg/kg daily) for 3 weeks, and EPCs numbers were determined. As shown in Fig. 8, fluvastatin treatment led to a more than 3-fold increase in DiLDL/lectin-positive cells, thus extending the \textit{in vitro} data.

3 DISCUSSION

The clinical benefit of statins therapy is primarily attributed to its LDL-lowering and potentially HDL-elevating effects. However, a subgroup analysis of large clinical trials indicates that statins-treated individuals have significantly less cardiovascular diseases than patients with comparable serum cholesterol levels\textsuperscript{20-22}. Subsequently, intriguing experimental data have shown that statins exhibit pleiotropic effects that can beneficially impact occlusive vascular diseases, including inhibition of smooth muscle proliferation and platelet aggregation, enhancement of endothelial...
function, and antiinflammatory actions[23-28]. Moreover, Statins have recently been shown to promote neovascularization and reendothelialization, which have been documented at least in part from the contribution of EPCs[9,14].

The results of the present study demonstrated for the first time that fluvastatin could augment EPC number, promote EPC proliferation, migration and adhesion in vitro as well as in vivo. The effects of fluvastatin on EPCs were comparable to those of simvastatin at the same concentration. These data were in line with a recently performed clinical study that demonstrated an increase in EPC number with enhanced migratory activity by atorvastatin treatment in patients with stable CAD[16]. The improvement of EPC function by statins may further contribute to neovascularization after ischemia. In addition, Llevadot et al. have recently documented that simvastatin can promote EPC proliferation, migration, and cell survival in vitro[14]. Dimmelber et al. also reported that simvastain, mevastatin and atorvastatin could induce the differentiation of endothelial progenitor cells and upregulate EPC numbers in vitro and in vivo[15]. Thus, our results, together with the findings of other investigators suggested a novel mechanism of action of statins: the augmentation of EPCs with enhanced functional activity.

There is strong evidence that EPCs play a significant role in neovascularization and reendothelialization, particularly during ischemic condition. More recently, 2 groups have documented in animals and human subjects that EPCs contribute up to 25% of endothelial cells in newly formed vessels[29,30]. Thus, increasing the number of circulating EPCs by transplantation of hematopoietic stem cells or by injection of in vitro-differentiated EPCs has been shown to improve neovascularization of ischemic hindlimbs[31,32], accelerate blood flow in diabetic mice[33], and improve cardiac function[34]. Moreover, Vasa et al. have recently reported that patients with CAD revealed reduced levels and functional impairment of EPCs, which correlated with risk factors for CAD[11]. Therefore, the stimulation of mobilization and/or differentiation of EPCs may provide a useful novel therapeutic strategy to improve postnatal neovascularization and reendothelialization in patients with CAD. However, due to the limited number of EPCs in the circulating blood (500~1000/ml), ex vivo expansion of EPCs appears to be necessary. Findings of us and other investigators have documented that statins can augment EPCs numbers and enhance EPCs functional activity[14-17]. Thus, the augmentation of EPC numbers by statins, which also regulate EPC differentiation, may be a novel strategy to improve neovascularization after ischemia, thereby, provide a therapeutic concept to improve EPC numbers and functions in patients with CAD.

The mechanisms mediating the effects of statins on EPC remain to be determined. It might be possible that statins induced EPC differentiation via the PI 3-kinase/Akt (PI3K/Akt) pathway as confirmed by the inhibitory effect of pharmacological PI3K blockers or overexpression of a dominant negative Akt construct[14,15]. Moreover, Assmus et al. have recently reported that HMG-CoA reductase inhibitors modulated expression of cell cycle genes including upregulation of cyclins (such as cyclins A, D, and F) and downregulation of the cell cycle inhibitor p27Kip1 in EPCs via the PI3K/Akt pathway, thus reduced senescence and increased proliferation of EPCs. In addition, they have demonstrated that statins inhibited senescence of EPCs independent of NO, reactive oxygen species, and Rho kinase, but dependent on geranylgeranylation phosphatase[17].

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