Effects of Atorvastatin on Angiogenesis in Hindlimb Ischemia and Endothelial Progenitor Cell Formation in Rats

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Aim: To investigate the mechanisms underlying the pro-angiogenic effects of statin, the effects of atorvastatin were investigated on the expression of angiogenic factors in ischemic hindlimbs of rats. The function and number of endothelial progenitor cells (EPCs) were investigated in hypertensive rats.

Methods: Hindlimb ischemia rats were administered 10 or 30 mg/kg/day atorvastatin orally for 2 weeks. Angiogenesis was evaluated by a laser Doppler and by Isolectin-B4 immunostaining. The expressions of VEGF, IL-8, angiopoietin (Ang)-1, Ang-2, eNOS, and hemoxidase (HO)-1 were evaluated by Western blotting and immunohistochemistry. Spontaneously hypertensive rats (SHR) were administered 10 mg/kg/day atorvastatin. EPC function was evaluated by colony formation and migration. The EPC number was evaluated by CD34-positive cells.

Results: A low dose of atorvastatin, but not a high dose, significantly increased regional blood flow. Atorvastatin significantly increased the expressions of VEGF, IL-8, Ang-1, Ang-2, eNOS, and HO-1 proteins in ischemic hindlimbs. Atorvastatin significantly increased the number and colony formation of EPCs and decreased oxidation in mononuclear cells from SHR.

Conclusion: Atorvastatin strongly induced angiogenesis with increases in angiogenic cytokines, HO-1 and EPC numbers. Statins are thus considered potential agents for therapeutic angiogenesis.


Key words: Statin, Angiogenesis, eNOS, Endothelial progenitor cell, Oxidation

Introduction

The number of patients presenting with severe limb ischemia, for which amputation is indicated, has increased in recent years, reflecting the aging population and dietary changes. Alternative therapy includes the attempt to develop new collateral vessels.

Bone marrow (BM) contains abundant stem cells and endothelial progenitor cells (EPCs). Autologous BM cell implantation has been practically applied for patients with limb ischemia. Investigators for the Therapeutic Angiogenesis using Cell Transplantation (TACT) study reported that BM cell implantation improves ischemic ulcers in limb ischemia by improving blood flow.

The mechanisms of angiogenesis by BM cell implantation had been thought to be due to implanted cell differentiation in the vessels; however, it has shifted to the overexpression of selected angiogenic factors that promote angiogenesis in the ischemic region. In addition, practical problems have been indicated for...
therapeutic angiogenesis by BM cell implantation. It requires the extraction of a large amount of BM under general anesthesia, which is risky for severe patients with cardiovascular diseases. The period for maintaining efficient blood flow is also limited; therefore, non-invasive and sustained treatments are required for therapeutic angiogenesis.

Circulating EPCs act to repair the endothelial damage which occurs in cardiovascular diseases. An impairment in EPC colony formation is associated with the incidence of cardiovascular diseases. Vas et al. documented an inverse correlation between the number of EPCs and the risk factors for coronary artery disease. The lifespan of stem cells and EPCs has been reported to be shortened by oxidative stress. It is therefore possible that oxidative stress induces endothelial damage by the suppression of EPC number and function. Antioxidative agents exert cardiovascular protective effects. Angiotensin converting enzyme inhibitors, angiotensin receptor blockers (ARB) and statins all appear to lower oxidative stress, and have even been advocated in cardiovascular protection. ARBs improve impaired EPC function by suppressing cardiovascular oxidation in spontaneously hypertensive rats (SHR). Statins have pleiotropic effects independent of their cholesterol-lowering effects. A large-scale clinical examination (ASCOT-LLA) showed that atorvastatin markedly reduced the incidence of stroke in patients with essential hypertension with normal cholesterol, thus suggesting the strong vascular protective effects of statin. Statins also induce strong pro-angiogenic effects. It is therefore possible that statins will provide feasible long-term angiogenic therapy instead of angiogenic cell transplantation.

The current study examined the effects of atorvastatin on the expression of angiogenic factors in an ischemic hindlimb and on EPC number and function in SHR.

**Methods**

**Ethics**

This study conformed to the standards of the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). All animals were handled in accordance with the guidelines of the Animal Care and Use Committee at Nihon University School of Medicine (Itabashi Tokyo) and the Law (No. 105) and Notification (No. 6) of the Japanese Government.

**Experimental Designs**

Male Wistar rats (Japan SLC, Shizuoka, Japan), weighing 280 to 320 g, were anesthetized with pentobarbital i.p. and subjected to hindlimb ischemia by resection of the left femoral artery. Atorvastatin was purchased from Pfizer Inc. (New York, NY, U.S.A.). After resection of the femoral artery, 10 or 30 mg/kg/day atorvastatin, dissolved in 0.5% methylcellulose, was administered orally for 2 weeks to rats with an ischemic hindlimb. Control rats were administered only methylcellulose.

To evaluate the effects of atorvastatin on EPC formation, SHR/Izm (Japan SLC) were administered 10 mg/kg/day atorvastatin orally for 2 weeks.

**Measurement of Arterial Blood Flow**

A laser Doppler flow probe was placed on the surface of the left foot to measure skin blood flow with a laser Doppler flow meter (PeriScan PIM II, Tokyo, Japan). The right foot served as a control.

**Histology of Neovascularization**

Ischemic hindlimb rats were killed 2 weeks after the start of atorvastatin administration. Leg skeletal muscles were fixed in 10% formalin. Paraffin-embedded sections were stained with hematoxylin. Biotinylated isolectin B4 (Vector Laboratories, Burlingame, CA, U.S.A.) and an avidin-biotin horseradish peroxidase (Vector Laboratories) with diaminobenzidine was used for microvessel immunostaining.

**Western Blot Analysis**

Tissue samples from ischemic hindlimb muscles were homogenized in 500 µL buffer (200 mmol/L sucrose and 20 mmol/L HEPES [pH 7.4]). Proteins were separated by electrophoresis in denaturing 12% polyacrylamide gels and then blotted onto a nitrocellulose sheet (Amersham Bioscience, Piscataway, NJ, U.S.A). Antibodies against vascular endothelial growth factor (VEGF), interleukin (IL)-8 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A), angiopoietin (Ang)-1, Ang-2 (Alpha Diagnostic International, San Antonio, TX, U.S.A), hemoxidase (HO)-1 and endothelial nitric oxide synthase (eNOS, Santa Cruz) were used at a dilution of 1:2000.

**Immunohistochemistry**

Sections were stained with primary antibody (anti-VEGF or anti-IL-8; Santa Cruz, or Ang-1; Alpha Diagnostic International) diluted 1:200 and then stained with the appropriate biotinylated secondary antibody. The reaction was visualized with ABC reagent (Santa Cruz). Nuclei were stained with Hoechst 33342.
Immunohistochemistry quantification was performed by a digital computer-assisted method as semiquantitative analysis\(^{19}\).

**EPC Colony Formation Assay**

A modified EPC colony formation assay was performed as previously described\(^{7, 12}\). Mononuclear cells (MNCs) were separated from rat peripheral blood using a Histopaque-1083 density gradient medium (Sigma-Aldrich, St. Louis, MO, U.S.A), and then suspended in 1 mL EGM-2 medium (Clonetics Corp, San Diego, CA, U.S.A). MNCs were inoculated into 6-well plates (5 \( \times 10^6 \) cells/well) and cultured for 24 hours. Nonadherent MNCs were reinoculated into vitronectin-coated plates (2 \( \times 10^6 \) cells/well) and cultured in a CO\(_2\) incubator at 37\(^\circ\)C for 7 days. The average number of colonies was calculated manually under a microscope.

**Flow Cytometric Analysis for EPC**

The number of circulating EPCs in peripheral blood was determined using a cell surface antigen as previously established\(^{20}\). MNCs with CD34\(^+\) were quantified as tentative EPCs. MNCs were stained with a fluorescein isothiocyanate (FITC)-conjugated anti-CD34 monoclonal antibody (Becton-Dickinson, Franklin Lakes, NJ, U.S.A.). Samples were subjected to 2D side scatter-fluorescence dot plot analysis (FACScan, Becton-Dickinson)\(^{21}\).

**EPC Migration Assay**

To investigate EPC migration activity, a modified Boyden chamber assay was performed in a 96-well microchemotaxis chamber (Neuroprobe Inc., Gaithersburg, MD, U.S.A.) as previously described\(^{22}\).

**Thiobarbituric Acid Reactive Substance (TBARS) Assay**

TBARS in MNCs were measured with a commercial kit (Oxi-Tek TBARS assay kit; Zeptometrix, Buffalo, NY, U.S.A.) as described previously\(^{23}\). The concentration of TBARS was expressed in pmol/106 cells by interpolation from a standard curve of malondialdehyde (MDA) at concentrations of 0–200 pmol/L.

**Statistical Analysis**

The results are shown as the mean ± SE. The significance of differences between mean values was evaluated by Student’s \( t \)-test for unpaired data and by two-way analysis of variance (ANOVA) followed by Duncan’s multiple range test.

**Results**

**Effect of Atorvastatin on Blood Flow and Neovascularization in Ischemic Hindlimb**

Low-dose (10 mg/kg/day) atorvastatin for 2 weeks significantly (\( p < 0.05 \)) increased blood flow at 2 and 4 weeks in comparison to control rats; however, high-dose (30 mg/kg) atorvastatin did not affect blood flow.
in comparison to control rats. Based on these results, a dose of 10 mg/kg/day atorvastatin was used to investigate the mechanisms of angiogenic effects in the following experiments (Fig. 1A).

The number of isolectin B4-positive vessels was significantly \( p < 0.05 \) increased in ischemic hindlimbs with 10 mg/kg/day atorvastatin for 2 weeks in comparison to the controls (Figs. 1B, C). These results suggest that atorvastatin actually induced neovascularization in ischemic hindlimbs.

**Effect of Atorvastatin on the Expression of Angiogenic Factors in the Ischemic Hindlimb**

Treatment with atorvastatin significantly \( p < 0.05 \) increased the abundance of VEGF, IL-8, Ang-1, Ang-2, and eNOS proteins in an ischemic hindlimb in comparison to the controls (Figs. 2, 3). In an immunohistochemical study, atorvastatin significantly \( p < 0.01 \) increased the expressions of VEGF, IL-8, and Ang-1 in an ischemic hindlimb in comparison to the controls (Figs. 4A, B). In addition, atorvastatin significantly \( p < 0.05 \) increased the abundance of HO-1 protein in an ischemic hindlimb in comparison to the controls (Fig. 3).

**Effects of Atorvastatin on EPC Formation and Oxidation in SHR**

Two weeks of treatment with atorvastatin did not affect blood pressure in SHR. Treatment with atorvastatin significantly \( p < 0.01 \) increased EPC colony formation (Fig. 5A), and significantly \( p < 0.05 \) increased the circulating number of EPCs (Fig. 5B) in SHR. Treatment with atorvastatin did not significantly change EPC migration in SHR (Fig. 5C). Atorvastatin...
significantly \( p < 0.05 \) decreased the TBARS score in MNCs from SHR (Fig. 5D). These results indicate that atorvastatin increased the number and formation of EPCs with oxidation inhibition in SHR.

**Discussion**

The present study confirmed the presence of neovascularization in the ischemic hindlimbs of rats treated with atorvastatin. Atorvastatin significantly increased the expressions of angiogenic cytokines, such
as VEGF, IL-8, Ang-1 and Ang-2, in an ischemic hindlimb. The increases in multiple angiogenic cytokines indicated that atorvastatin might protect ischemic hindlimb tissue, and these cytokines might be subsequently increased. Moreover, treatment with atorvastatin increased eNOS production in the ischemic hindlimb. Laufs et al. previously demonstrated that statins improve angiospasms of atherosclerotic coronary arteries by the stabilization of eNOS mRNA while decreasing RhoA activity; therefore, the increases in angiogenic cytokines and eNOS potentially induced neovascularization in ischemic hindlimbs in the present study.

Interestingly, low-dose (10 mg/kg) atorvastatin, but not a high dose (30 mg/kg), increased regional blood flow in ischemic hindlimbs in the present study. Statins have been reported to exert biphasic angiogenic effects in vitro and in vivo. A low-dose of statin induces angiogenesis, while a high-dose inhibits it. Sumi et al. reported that a low-dose of fluvastatin upregulates eNOS and suppresses superoxide anions in the arterial endothelium to improve the bioavailability of NO. In addition, according to the review report from Pharmaceuticals and Medical Devices Evaluation Center in Japan, dated December 24, 1999, the non-observed adverse effect level of atorvastatin was 20 mg/kg/day for male rat fertility. It is possible that the high dose was toxic in the present experiments. It is thought, therefore, that the evaluation of suitable doses of statin will be important for angiogenesis and cardiovascular protection in clinical applications.

In the present study, atorvastatin potentially increased the EPC number and colony formation, but did not increase EPC migration, with suppression of oxidative stress in SHR. This suggests that atorvastatin mainly increases the EPC number. Statins have been shown to induce the mobilization and differentiation of EPCs, leading to accelerated angiogenesis and improved endothelialization. It is possible therefore that atorvastatin may directly affect the BM to mobilize EPCs to the peripheral blood. The functional improvement and increased homing capacity of EPCs in response to statin treatment might reverse the impaired functional regeneration capacities seen in patients with risk factors for coronary artery diseases. The eNOS expression and functional integrity have been reported to be pre-requisites for statin-mediated EPC mobilization. In the present study, treatment with atorvastatin significantly increased the expression of eNOS; therefore, statin-induced eNOS possibly improved EPC formation and induced angiogenesis in the present study.

Moreover, treatment with atorvastatin significantly inhibited the oxidation of MNCs in SHR. Oxidative stress has been reported to accelerate the senescence of stem and progenitor cells. It is thought that statin-induced EPC formation is due to the antioxidative effects of statin. The effects of ARBs on EPC function and cardiovascular oxidation were recently investigated in stroke-prone SHR. EPC colony formation was markedly suppressed with increases in oxidation, and treatment with ARBs markedly increased EPC colony formation with decreases in NAD(P)H oxidase. This suggested that tissue angiotensin II increased oxidation by NAD(P)H oxidase, which...
decreased EPC formation. In the present study, treatment with atorvastatin significantly increased HO-1 levels in the ischemic hindlimb. HO-1 is a strong antioxidative molecule. Statins have been reported to increase HO-1 production to inhibit oxidation in vivo. Hsu et al. demonstrated that oral administration of statins increased HO-1 production in the mouse liver, lung, brain and heart and suggested that the anti-oxidative effects of statins in cardiovascular tissues are strongly induced by HO-1. HO-1 is a stress-inducible enzyme that regulates angiogenesis through the induction of VEGF, SDF-1, and IL-8. Thus, increases in HO-1 production in the ischemic hindlimb with atorvastatin might improve ischemia-induced muscle injury and subsequently increase the production of VEGF, IL-8, Ang-1 and Ang-2 to induce the neovascularization. Increases in HO-1 with atorvastatin also induce anti-oxidation in the whole body, which may increase the formation and number of EPCs in SHR, an oxidative animal model.

Conclusions

In total, the present study revealed that atorvastatin stimulates eNOS and HO-1 production, which may induce injured skeletal muscle to increase angiogenic cytokines. Atorvastatin also stimulates the mobilization of EPCs into the peripheral blood. In concert with these mechanisms, atorvastatin might induce angiogenesis. Thus, statins have the potent pro-angiogenic effects, which could provide convenient and feasible angiogenic therapy in drug-induced regenerative medicine.

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