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Fluvastatin accelerates re-endothelialization impaired by local sirolimus treatment

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ABSTRACT

Sirolimus-eluting stent reduces restenosis after percutaneous coronary intervention. However, accumulating evidence suggests that sirolimus potentially affects re-endothelialization, leading to late thrombosis. Statins have protective effects on endothelium. Recently, statins are reported to increase the number of circulating endothelial progenitor cells (EPCs) and accelerate re-endothelialization after vascular injury. Here, we tested the hypothesis that fluvastatin has beneficial effect on re-endothelialization after local sirolimus treatment. We performed wire-mediated vascular injury to both sides of femoral arteries of wild-type mice and bone marrow chimeric mice. Either sirolimus (100 μg) or DMSO was administered locally to the perivascular area of the injured arteries. All mice received either fluvastatin (5 mg/kg/day) or vehicle by gavage starting at one week before the surgery until sacrifice. At 4 weeks after the surgery, re-endothelialization of the sirolimus-treated artery was significantly less than that of DMSO-treated one in the vehicle-treated mice as determined by the percentage of CD31-positive area ($P<0.05$). Systemic administration of fluvastatin accelerated the re-endothelialization in the sirolimus-treated artery to the similar degree of that in the DMSO-treated artery ($P=$ NS). Contribution of bone marrow-derived cells to re-endothelialization was seldom observed in bone marrow chimeric mice regardless of fluvastatin administration. Fluvastatin significantly ameliorated proliferation (2.5-folds) and migration activities (2.3-folds) of mature endothelial cells impaired by sirolimus treatment ($P<0.05$, respectively). Fluvastatin increased endothelial nitric oxide synthase expression and decreased plasminogen activator inhibitor-1 expression in mature endothelial cell in the presence of sirolimus ($P<0.05$, respectively). Our findings suggest that fluvastatin has protective effects against impaired re-endothelialization after sirolimus treatment.

1. Introduction

Sirolimus-eluting stent is remarkably effective in reducing restenosis after percutaneous coronary intervention (Marks, 2003; Moses et al., 2003). Several studies showed its excellent long-term results with regard to clinical and angiographic outcomes (Fajadet et al., 2005; Serruys et al., 2002). However, concern has been raised that sirolimus-eluting stent might be associated with increased rate of late thrombosis (Iakovou et al., 2005; Jeremias et al., 2004; McFadden et al., 2004). Stent implantation causes endothelial denudation, medial dissection and exposure of the subintima, which leads to subsequent platelet activation. It was hypothesized that sirolimus-eluting stent might retard re-endothelialization. Sirolimus was reported to up-regulate thrombotic genes in endothelial cells (Finn et al., 2007; Muldowney et al., 2007). Recently, it was demonstrated that sirolimus-eluting stent implantation leads to endothelial dysfunction (Hofma et al., 2006), causing impaired collateral flow (Meier et al., 2007) and paradoxical vasoconstriction in the adjacent segment (Togni et al., 2005). Hence, augmentation of re-endothelialization and amelioration of endothelial dysfunction represent effective strategy to overcome potential unfavorable effects associated with local sirolimus treatment.

Endothelium plays a major role in maintenance of vascular homeostasis, including regulation of vascular tone, remodeling, thrombogenesis and fibrinolysis (Lamb and Barna, 1998; Luscher and Noll, 1995). It is a widely accepted view that damages to the endothelium perturb the homeostasis and initiate atherosclerosis process including endothelial permeability, platelet aggregation, leukocyte adhesion, and generation of cytokines (Ross, 1999). Bone marrow-derived endothelial progenitor cells (EPCs) as well as preexisting endothelial cells have been suggested to contribute to preservation of endothelial integrity (Asahara et al., 1997; Kong et al., 2004). Statins have beneficial effects on not only mature endothelial cells (Wolfrum et al., 2003) but also EPCs (Dimmeler et al., 2001; Walter et al., 2004; Werner et al., 2002). Therefore, we hypothesized that statins may improve the impaired re-endothelialization after sirolimus treatment.

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To test this hypothesis, we examined re-endothelialization of injured artery after sirolimus treatment. We also evaluated the effects of fluvastatin on EPCs and human umbilical vein endothelial cells (HUVECs) after sirolimus treatment. Results suggest that fluvastatin accelerates delayed re-endothelialization with beneficial effects on mature endothelial cells.

2. Materials and methods

2.1. Animals

Wild-type C57BL/6 mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Transgenic mice (C57BL/6 background) that ubiquitously express enhanced green fluorescent protein (GFP-mice) were a generous gift from Dr. Masaru Okabe (Osaka University, Osaka, Japan)(Okabe et al., 1997). All mice were kept in microisolator cages on a 12-h day/night cycle and fed on regular chow. All experimental procedures and protocols were approved by the Animal Care and Use Committee of the University of Tokyo and complied with the "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 86-23, revised 1985).

2.2. Vascular injury and local delivery of sirolimus

Bone marrow transplantation was performed at eight weeks of age as already described (Sata et al., 2002). Only bone marrow chimeric mice with more than 80% chimerism were used. Both femoral arteries of 8-week-old male C57BL/6 mice and 18-week-old female BMT mice were injured by inserting a straight spring wire (0.38 mm in diameter, No. C-SF-15-15, COOK) as already described (Sata et al., 2002). Sirolimus was suspended in DMSO at a concentration of 100 μg/μl. After vascular injury, 100 μg of sirolimus suspended in 50 μl of 20% Pluronic F-127 gel (Sigma-Aldrich) was administered to the perivascular area of left femoral artery, while the same volume of Pluronic gel containing only DMSO was administered around the right femoral artery. Fluvastatin (5 mg/kg/day) or vehicle was administered everyday by gavage starting at one week before the injury until the harvest. All animals were randomly assigned to receive either fluvastatin or vehicle. At 4 weeks after the injury, animals were euthanized for analyses.

2.3. Immunohistochemistry

Four weeks after the surgery, mice were sacrificed with overdose of pentobarbital, and perfused at a constant pressure via the left ventricle with 0.9% sodium chloride solution. Then, perfusion fixation with methanol was performed. The injured arteries were excised and were further fixed in methanol, then embedded in paraffin. Thin sections (4 μm) were used for immunohistochemistry. To detect endothelium, sections were incubated with an anti-CD31 antibody (clone MEC13.3, BD Biosciences) followed by the avidin–biotin complex technique and stained with Vector Red substrate kit (Vector). Each section was counterstained with hematoxylin. Morphometric analysis was performed using image analysis software (Image-Pro Plus version 4.5, Media cybernetics) as described previously (Sata et al., 2002). To examine the degree of re-endothelialization, the percentage of CD31-positive luminal side was measured. Intima/media ratio was also calculated. Measurement was performed on three different sections and the measured values were averaged in each artery. To examine the contribution of bone marrow-derived cells to re-endothelialization, we also performed immunofluorescence double staining using the samples from the bone marrow chimeric mice. Sections were stained with primary antibodies (anti-CD31 antibody and anti-GFP antibody (Molecular Probes)) followed by Cy3-conjugated anti-rat Ig (Jackson) and Alexa Fluor 488-conjugated anti-rabbit Ig (Molecular Probes) antibodies. Nuclei were counterstained with Hoechst 33258. The sections were mounted with the Prolong Antifade Kit (Molecular Probes) and observed under a confocal microscope (FLUOVIEW FV300, Olympus).

2.4. FACS analysis

At the time of sacrifice, peripheral blood was collected from heart to heparin-containing tube. Red blood cells were removed using PharM Lyse (BD Biosciences), then white blood cells were stained with FITC-conjugated anti-CD34 antibody (BD Pharmingen), PE-conjugated anti-Flk-1 antibody (BD Biosciences) and biotin-conjugated anti-CD133 (eBiosciences) antibody followed by streptavidin-PE/TexasRed (BD Pharmingen). Dead cells were excluded with 7AAD (BECKMAN COULTER) staining. Percentage of $CD34^{-}/CD133^{+}/Flk\text{-}1^{+}$ population was analyzed by flowcytometry (EPICS XL, BECKMAN COULTER) (Friedrich et al., 2006).

2.5. Culture of EPCs

Peripheral mononuclear cells were isolated from the peripheral blood of healthy human volunteers by density gradient centrifugation with HISOPAQUE-1077 (Sigma). Mononuclear cells were cultured at a density of 1×10^{6} cells per fibronectin-coated well in a 96-well dish in EBM (Clonetics) supplemented with 1 μg/ml hydrocortisone, 3 μg/ml bovine brain extract, 10 ng/ml vascular endothelial growth factor and 20% FBS. Sirolimus (WAKO) was added to the culture medium at a concentration of 0.1 ng/ml. At 4, 8 and 12 days, the culture medium was changed and non-adherent cells were removed. To identify endothelial cell-like cells, adherent cells were incubated with 10 μg/ml acetylated low density lipoprotein labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyamine perchlorate (DiI-Ac-LDL) (Biomedical Technologies Inc.) for 4 h. Cells were washed in PBS and fixed with 4% formaldehyde, and counterstained with FITC-labeled lectin from *Bandeiraea simplicifolia* (FITC-*BS* lectin, Sigma). Cells that were positive for both DiI-Ac-LDL and FITC-*BS*-lectin were judged as endothelial-like cells as previously described (Fukuda et al., 2005).

2.6. Assessment of cell viability

HUVECs were purchased from Clonetics and cultured in EGM-2. HUVECs were seeded at a density of 3×10^{3} cells per well in 96-well plates in EGM-2. Sirolimus (10 ng/ml) was added to the medium following pre-incubation with fluvastatin (100 nM) for 2 h. After 24 h, MTS assay was performed according to the manufacturer's instructions (Promega) (Sata et al., 2000).

2.7. Scratch injury model in HUVEC monolayer

To determine the migration activity of endothelial cells, HUVECs were seeded onto 6-well cell culture plates at 80–90% confluency in EGM-2. After pre-treatment with 100 nM of fluvastatin for 2 h, scratch injury was applied using a disposable surgical scalpel. After injury, the monolayer was gently washed with PBS. The medium was replaced with EGM-2 in the presence of fluvastatin (100 nM) and sirolimus (10 ng/ml) for 18 h. Cell migration from the edge of the injured monolayer was quantified by measuring distance between the wound edges as already described (Weis et al., 2002).

2.8. Real-time PCR

To investigate the effects of fluvastatin on endothelial function, we performed real-time PCR. Both HUVECs and endothelial-like cells were treated with or without 100 nM fluvastatin for 2 h in EBM containing 1% FBS, and then incubated with 10 ng/ml of sirolimus for 4 h. Total RNA was extracted using total RNA extraction kit (Agilent