Potent Inhibitory Effect of Sirolimus on Circulating Vascular Progenitor Cells

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Background—Neointimal hyperplasia is the major cause of in-stent restenosis (ISR). The sirolimus-eluting stent (SES) has emerged as a promising therapy to prevent ISR; however, the exact mechanism by which locally delivered sirolimus, an immunosuppressive agent, prevents ISR remains unknown. Recent evidence suggests that circulating progenitor cells may contribute to neointimal formation.

Methods and Results—Mononuclear cells (MNCs) were isolated from peripheral blood of healthy human volunteers. Smooth muscle (SM)–like cells outgrew from the culture of MNCs (1×10⁶) in the presence of platelet-derived growth factor-BB and basic fibroblast growth factor, whereas endothelial cell–like cells were obtained in the presence of vascular endothelial growth factor. Sirolimus potently inhibited SM-like cell outgrowth. The number of SM-like cells was significantly reduced at a concentration as low as 0.1 ng/mL (15.9±5.8% of control, P<0.001). Sirolimus also exerted an inhibitory effect on endothelial cell–like cells that originated from MNCs. Wire-mediated vascular injury was induced in femoral arteries of bone marrow chimeric mice. Either vehicle or sirolimus was administered locally to the perivascular area of the injured arteries. Sirolimus significantly reduced neointima hyperplasia at 4 weeks (intima/media ratio 2.0±0.3 versus 1.0±0.2, P<0.05) with a decreased number of bone marrow–derived SM-like cells and hematopoietic cells in the lesion. Reendothelialization was retarded in the arteries treated with sirolimus.

Conclusions—The potent inhibitory effects of sirolimus on circulating smooth muscle progenitor cells may mediate the clinical efficacy of SES, at least in part. Sirolimus potentially may affect reendothelialization after stent implantation.

Key Words: stents ■ restenosis ■ balloon ■ atherosclerosis ■ endothelium

Implantation of coronary stents has been shown to reduce the risk of periprocedural complications and restenosis more than balloon angioplasty alone.¹ However, in-stent restenosis (ISR) remains a significant clinical problem limiting the long-term success of percutaneous treatment.¹ The principal cause of ISR is neointimal hyperplasia resulting from the excessive accumulation of smooth muscle cells (SMCs). Recently, the sirolimus-eluting stent (SES) has emerged as a promising strategy to prevent ISR.²,³ A randomized, double-blind trial demonstrated that the use of SES reduced the rates of restenosis and clinical events even in patients with complex lesions.³ Despite increasing clinical interest in SES, very little is known about the mechanism by which locally delivered sirolimus, a hydrophobic macrolide with potent immunosuppressive activity, prevents ISR.²

We and others suggested that bone marrow (BM) cells give rise to vascular progenitor cells that home in on injured arteries and differentiate into SMCs or endothelial cells (ECs), thereby contributing to vascular repair and lesion formation.⁴⁻⁹ Here, we investigated the effects of sirolimus on human vascular progenitor cells that may contribute to neointimal hyperplasia and reendothelialization after stent implantation. The in vivo effect of sirolimus was also evaluated in a model of neointimal hyperplasia with bone marrow chimeric mice. Results suggest that sirolimus has a potent inhibitory effect on both smooth muscle progenitor cell and endothelial progenitor cell incorporation at the sites of vascular lesions.

Methods

Cell Culture

Peripheral mononuclear cells (MNCs) were isolated from blood of healthy human volunteers by density gradient centrifugation with HISOPAQUE-1077 (Sigma). MNCs were cultured at a density of 1×10⁶ cells per fibronectin-coated well in a 96-well dish in HuMedia-SG2 (KURABO) supplemented with 10 ng/mL platelet-derived growth factor (PDGF)-BB and 10 ng/mL basic fibroblast growth factor (smooth muscle progenitor cell medium). The same number of MNCs were cultured 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 (endothelial progenitor cell medium). Sirolimus (Wako Pure Chemical) was added to the culture medium at a concentration of 0.1 to 100 ng/mL. At 4, 8, and 12 days, the culture medium was changed, and...
nonadherent cells were removed. Jurkat cells and Sertoli cells were purchased from the American Type Culture Collection (Rockville, Md). Human aortic SMCs (HASMCs) were purchased from KURABO and cultured in HuMedia-SG2. Human umbilical vein ECs (HUVECs) were purchased from Clonetics and cultured in EGM-2 (Clonetics). HASMCs or HUVECs were seeded at a density of 5 x 10^4 cells per well in 24-well plates. After culture for 72 hours in the presence of sirolimus, cells were counted.

**Immunocytochemistry**

To identify smooth muscle (SM)-like cells, adherent cells were fixed at 14 days in 4% paraformaldehyde and permeabilized with 0.5% NP40. After blocking with 1% goat serum, cells were incubated with an alkaline phosphatase–conjugated anti-α-smooth muscle actin (α-SMA) antibody (Sigma) and stained with a Vector red substrate kit (Vector). To identify EC-like cells, adherent cells were incubated with 10 µg/mL acetylated LDL labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindifluorocarbonyl carbazole perchorl (Di-AC-LDL; Biomedical Technologies Inc) for 4 hours. Cells were washed in PBS, fixed with 4% formaldehyde, and counterstained with FITC-labeled lectin from Bandeiraea simplicifolia (FITC-BS lectin, Sigma). Cells that were positive for both Di-AC-LDL and FITC-BS lectin were deemed EC-like cells, as described previously.8

**Reverse Transcription–Polymerase Chain Reaction**

Total RNA was prepared with RNAzol reagent (Tel-Test, Inc). Reverse transcription was performed with 1 µg of RNA, random hexamer primers, and MMLV reverse transcriptase (ReverTraAce-α) (Toyobo). Polymerase chain reaction (PCR) primers were as follows: α-SMA 5'-CCGCTAGTCTGGAAGAG-3' and 5'-GTGACTCTCTCCTGATCGTTG-3'; GAPDH 5'-ACACAGTCCATCAGCATCAC-3' and 5'-TCCACCCGTTGCTGTA-3'. PCR reactions were performed for 30 cycles of 30-second denaturation at 94°C, 30 seconds of annealing at 60°C, and 1 minute of extension at 72°C followed by 10 minutes of final extension. PCR for FK506-binding protein 12 (FKBP12) was performed with a primer set of 5'-GAGAAAGACGCACATAGAC-3' and 5'-TCTAGAATCTGATGGGAG-3' for 30 cycles of denaturation (94°C, 15 seconds), annealing (48°C, 30 seconds), and polymerization (68°C, 45 seconds). After agarose gel (3%) electrophoresis in the presence of ethidium bromide, the PCR products were revealed by ultraviolet irradiation.

**Bone Marrow Transplantation**

LacZ mice, which are knock-in mice that express the LacZ gene in essentially all tissues (C57BL/6 x 129S background), were originally purchased from Jackson Laboratory (B6; 129S-Gtrosa26, stock No. 002073, Bar Harbor, Me). LacZ mice were maintained in our animal facility and intercrossed with C57BL/6J mice. The resulting litters were used for the present study.4 The homozygotes and wild-type mice were screened by PCR analysis of tail DNA according to the protocol provided by Jackson Laboratory. GFP mice, which are transgenic mice (C57BL/6 background) that ubiquitously express enhanced green fluorescent protein (GFP), were a generous gift from Dr Kazuhide Hasegawa (Kyowa Hakko, Japan). Bone marrow transplantation (BMT) was performed from LacZ mice to wild-type mice (BMTα-SMA-Wild) or GFP mice to wild-type mice (BMTGFp-Wild) as described previously.4,11 A total of 85% to 95% of peripheral leukocytes had been reconstituted as determined by in situ hybridization for Y chromosome (BMT Male LacZ/Female Wild mice) or flow cytometry (BMTGFp-Wild mice). All procedures that involved experimental animals were performed in accordance with protocols approved by the institutional committee for animal research 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).

**Vascular Injury and Local Delivery of Sirolimus**

Twenty weeks after BMT, both femoral arteries of BM chimeric mice were injured by inserting a straight spring wire (0.38 mm in diameter, No. C-SF-15-15, Cook) as described previously.12 Sirolimus was suspended in DMSO at a concentration of 25 µg/µL. After vascular injury, 25 µg (n = 8) of sirolimus suspended in 50 µL of 20% Pluronic F-127 gel (Sigma) was administered to the perivascular area of the right femoral artery in BMTα-SMA-Wild mice, and the same volume of Pluronic gel containing only DMSO was administered around the left femoral artery.

**X-Gal Staining and Morphometric Analysis**

Four weeks after surgery, mice were euthanized with an overdose of pentobarbital and perfused at a constant pressure via the left ventricle with 0.9% sodium chloride solution. The injured arteries were excised and stained with X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) as described previously.4,11 The arteries were embedded in paraffin, and cross sections were made as described previously.12 After counterstaining with hematoxylin, morphometric analysis was performed with image-analysis software (Image-Pro Plus version 4.5, Media Cybernetics, USA) as described previously.13 Intima/media ratio and the number of LacZ-positive cells were averaged on 3 different sections from each artery.

**Immunofluorescence Staining**

To study the effect of sirolimus on reendothelialization, cross sections were incubated with an anti-C3D3 antibody (clone MEC13.3, BD Biosciences) and Cy3-conjugated anti-α-SMA antibody (Sigma), followed by incubation with an FITC-conjugated anti-rat Ig secondary antibody (Jackson ImmunoResearch). To characterize the BM-derived cells observed in the neointima of BMTα-SMA-Wild mice, X-gal–stained cross sections were incubated with a Cy3-conjugated anti-α-SMA antibody and an anti-C4D4 antibody (BD Biosciences), followed by incubation with an FITC-conjugated anti-rat Ig secondary antibody. The femoral arteries harvested from BMTGFp-Wild mice were embedded in plastic resin (Technovit 8100, Heraeus Kulzer) as described previously.14 An anti-SM1 rat monoclonal antibody was kindly provided by Dr Kazuhide Hasegawa (Kyowa Hakko, Japan). Thin sections (4 µm) were incubated with Cy3-conjugated anti-α-SMA antibody or anti-SM1 antibody followed by Cy3-conjugated anti-rat Ig antibody. After immunofluorescence staining, nuclei were counterstained with Hoechst 33258 (Sigma). The sections were mounted with the Prolong Antifade Kit (Molecular Probes) and observed under a PROVIS AX80 microscope (Olympus) equipped with a mercury/halogen dual-illumination system and a charge-coupled device camera (DP50, Olympus). High-resolution fluorescence images were taken with a confocal laser scanning system (FLUOVIEW FV300, Olympus).

**Statistical Analysis**

Values are expressed as mean ± SEM for continuous variables. Comparisons of multiple groups were made by 1-way ANOVA, followed by Scheffé multiple comparison test. A probability value <0.05 was considered statistically significant.

**Results**

**Potent Inhibitory Effect of Sirolimus on SM Progenitor Cells**

Human peripheral MNCs were cultured in fibronectin-coated wells in the presence of PDGF-BB and basic fibroblast growth factor (SM progenitor cell medium). When the medium was changed at 4 days, there were adherent cells that consisted of heterogeneous cell types. Some cells remained round, whereas others showed a spindlelike shape and were displayed as a monolayer. At 14 days, cells with a polygonal shape were dominant. Consistent with a previous report,5 mRNA for FK506-binding protein 12 (FKBP12) was performed with a primer set of 5'-GAGAAAGACGCACATAGAC-3' and 5'-TCTAGAATCTGATGGGAG-3' for 30 cycles of denaturation (94°C, 15 seconds), annealing (48°C, 30 seconds), and polymerization (68°C, 45 seconds). After agarose gel (3%) electrophoresis in the presence of ethidium bromide, the PCR products were revealed by ultraviolet irradiation.
cells at 4 days; however, the number of α-SMA–positive cells at 14 days was dramatically decreased by sirolimus (Figure 1B and 1C). At a concentration as low as 0.1 ng/mL, sirolimus significantly reduced the number of α-SMA–positive cells to 15.9 ± 5.8% of control (P < 0.0001).

Effects of Sirolimus on Endothelial Progenitors, ECs, and SMCs

Sirolimus also inhibited the proliferation of HASMCs and HUVECs in a dose-dependent manner (data not shown); however, its inhibitory effect on these cells was relatively mild (Figure 2A). At a concentration as low as 0.1 ng/mL, sirolimus significantly reduced the number of α-SMA–positive cells to 15.9 ± 5.8% of control (P < 0.0001).

Effects of Locally Delivered Sirolimus on Neointimal Formation and Reendothelialization After Vascular Injury

The in vivo effects of sirolimus on vascular progenitor cells were studied in BM chimeric mice with a mouse model of vascular injury that induces reproducible neointima hyperplasia. At 4 weeks after injury, there was neointima hyperplasia that contained BM-derived LacZ-positive cells.
BMT-LacZ mice. Local delivery of sirolimus significantly reduced neointimal formation (intima/media ratio 2.0±0.3 versus 1.0±0.2, P=0.03; Figure 3A and 3B). The reduction in neointimal hyperplasia was associated with a decrease in the number of LacZ-positive cells in the neointima (number of LacZ-positive cells/neointima 20.2±4.1 versus 7.3±2.4, P=0.02). In the lesion treated with vehicle, 55.1±26.1% of the luminal side was coated with the regenerated endothelium as determined by anti-CD31 immunostaining. On the other hand, endothelium was detected only in 7.7±3.4% of the luminal side of the sirolimus-treated arteries. Taken together, these results indicate that locally delivered sirolimus possibly affects the reendothelialization process after severe vascular injury, although neointima hyperplasia is markedly attenuated (Figure 3C).

Characterization of BM-Derived Neointimal Cells
BM-derived cells observed in neointima were characterized by confocal immunofluorescence study. The wire injury induced vascular lesions that were predominantly composed of α-SMA–positive cells. In BMT-GFP→Wild mice, we readily detected GFP-positive intimal cells that were positive for α-SMA (Figure 4A). On the other hand, there were few neointimal cells that expressed both GFP and SM1, a marker for differentiated SMCs (Figure 4B). These results suggest that BM-derived α-SMA–positive cells do not become highly differentiated SMCs, at least at 4 weeks after wire injury. In BMT-LacZ→Wild mice, most of the BM-derived neointimal cells expressed α-SMA. There were BM-derived cells that expressed both α-SMA and CD45, a pan-hematopoietic marker (Table). Local administration of sirolimus potently reduced the number of LacZ+/α-SMA+ cells in the neointima (Figure 4C and 4D). Sirolimus also reduced the number of LacZ+/CD45+ cells. Reduction in the number of LacZ+ cells in neointima was associated with a reduction in the total number of α-SMA–positive neointimal cells (Figure 4E).

Discussion
It is assumed that sirolimus locally delivered from SES prevents neointimal hyperplasia by inhibiting migration and proliferation of SMCs that originate from the adjacent media. However, it was reported that medial SMCs express little FKBPU2, which sirolimus binds to prevent cell-cycle progression through the upregulation of the cyclin-dependent kinase inhibitor p27(S10). Thus, it is likely that sirolimus may also target cell types other than medial SMCs. Recent reports suggested that circulating progenitor cells may contribute to neointimal hyperplasia in models of transplant-associated arteriosclerosis, hyperlipidemia-induced atherosclerosis, and postangioplasty restenosis. It has been consistently reported that SM-like cells with specific growth, adhesion, and integrin profiles could grow from human peripheral MNCs. Moreover, a significant part of SMCs throughout the atherosclerotic vessel wall was shown to derive from donor BM in gender-mismatched BMT patients. Thus, it is possible that circulating cells may participate in the pathogenesis of human ISR.

In the present study, sirolimus potently inhibited MNC differentiation into SM-like cells. The inhibitory effect was achieved at a concentration as low as 0.1 mg/mL, at which sirolimus had no significant effect on the proliferation of HASMCs that expressed low FKBPU2. In fact, in our mouse model of severe vascular injury, locally derived sirolimus efficiently decreased the number of BM-derived cells in the neointima and attenuated neointima hyperplasia. These results suggest that the clinical efficacy of SES against restenosis might be achieved, at least in part, through its inhibitory effect on circulating SM progenitors.

In the present study, local administration of sirolimus also potently reduced the numbers of BM-derived CD45-positive hematopoietic cells in the neointima, which was associated with a significant reduction in the total number of α-SMA–positive cells in the neointima. It is possible that the inhibi-
Sirolimus also inhibited the proliferation of human vascular ECs at 1.0 ng/mL or higher concentrations. Furthermore, locally delivered sirolimus potentially affected reendothelialization after wire-mediated vascular injury. It was reported that a decrease in circulating endothelial progenitor cells is associated with a high incidence of cardiovascular disease. Thus, inhibition of endothelial progenitor cells may result in delayed reendothelialization and wound healing after stent implantation, which may lead to fatal late thrombosis. In fact, it was observed that oral administration of everolimus, a macrolide of the same family as sirolimus, results in delayed endothelial coverage over the stent surface with loose EC junction, although in-stent neointimal growth was suppressed. Moreover, there have been autopsy case reports that revealed late stent thrombosis or delayed reendothelialization more than 18 months after SES deployment.

There are controversies about the potential of BM cells to differentiate into other lineages. In the present study, BM-derived cells substantially contributed to neointimal formation after vascular injury, as reported by us and others. Many BM-derived cells in neointima expressed α-SMA as detected by an antibody (clone 1A4); however, we seldom detected BM-derived neointimal cells that were positive for SM1. There were α-SMA-positive cells that expressed CD45, the pan-hematopoietic marker. Thus, it might be a rare property of BM cells to transdifferentiate into fully differentiated SMCs in vascular lesions, at least at 4 weeks after injury, although a significant portion of the neointima could be formed by BM-derived SMC-like cells. Consistent with this notion, numerous reports have documented that neointimal SMCs are quite distinct from medial cells in morphology and gene expression. It was reported that putative circulating fibrocytes can express α-SMA after 3 weeks in culture. In conclusion, our results suggest that inhibitory effects on SM progenitor cells and on inflammatory cells may mediate the clinical efficacy of SES. We should be cautious about the potential deleterious effects of SES on reendothelialization.

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