

PRE-CLINICAL RESEARCH

Sirolimus and Everolimus Induce Endothelial Cellular Senescence Via Sirtuin 1 Down-Regulation

Therapeutic Implication of Cilostazol After Drug-Eluting Stent Implantation

Hidetaka Ota, MD, PhD, Masato Eto, MD, PhD, Junya Ako, MD, PhD, Sumito Ogawa, MD, PhD, Katsuya Iijima, MD, PhD, Masahiro Akishita, MD, PhD, Yasuyoshi Ouchi, MD, PhD

Tokyo, Japan

Objectives

The aim of this study was to compare the effects of paclitaxel, sirolimus, and everolimus on the senescent phenotype in human endothelial cells, and to further investigate possible involvement of mammalian sirtuin 1 (Sirt1) down-regulation as a mechanism.

Background

Endothelial cell senescence may play a role in impaired re-endothelialization after drug-eluting stent (DES) implantation. Recently, the down-regulation of Sirt1 has been shown to mediate oxidative stress-induced endothelial senescence.

Methods

Senescent human umbilical vein endothelial cells (HUVEC) were judged by senescence-associated β -galactosidase assay (SA- β gal), morphological appearance, and plasminogen activator inhibitor (PAI)-1.

Results

Treatment with paclitaxel, sirolimus, and everolimus significantly caused a senescent phenotype and PAI-1 up-regulation, associated with a decrease in endothelial nitric oxide synthase (eNOS) and Sirt1 expression. Overexpression of Sirt1 or Sirt1 activation reversed the sirolimus- or everolimus-induced senescent phenotype. Interestingly, paclitaxel-induced senescence was not suppressed by Sirt1 overexpression, suggesting the existence of a different mechanism. Cilostazol markedly inhibited the sirolimus- or everolimus-induced senescent phenotype (sirolimus or everolimus [2.5 nmol/l]; 49.2% or 53.0% SA- β gal positive vs. only 13.6% or 14.6% with cilostazol [100 μ mol/l]) and PAI-1 up-regulation, but had no influence on the effects of paclitaxel. Finally, aspirin significantly blunted sirolimus- or everolimus-induced senescence, but neither ticlopidine nor clopidogrel had any effects.

Conclusions

Sirolimus and everolimus induce endothelial senescence involving down-regulation of Sirt1. In contrast, the development of endothelial senescence by paclitaxel involves a Sirt1-independent pathway. Because sirolimus and everolimus are involved in Sirt1 modulation, cilostazol rescues HUVEC from sirolimus- or everolimus-induced senescence. These results may have therapeutic implications in the clinical sequelae after DES implantation. (J Am Coll Cardiol 2009;53:2298–305) © 2009 by the American College of Cardiology Foundation

Because of the marked reduction in restenosis rate, drug-eluting stent (DES) implantation has been the mainstay of percutaneous coronary interventions. Recent real-world experiences, however, have raised concern over a possible increase in the frequency of late stent thrombosis (ST), occurring more than 1 year after stent implantation (1). Pathological studies have shown that a lack or delay of endothelial healing plays an important role in the pathogenesis of ST (2).

Endothelial senescence has been proposed to be involved in endothelial dysfunction, atherogenesis, and thrombosis (3). Drugs used for DES, including paclitaxel and limus family members (e.g., sirolimus, everolimus), inhibit the growth of endothelial cells, possibly leading to endothelial senescence and delayed re-endothelialization. However, the effect of such drugs on endothelial cell senescence has not been well documented.

Mammalian sirtuin 1 (Sirt1), the closest homolog of silent information regulator 2, regulates the cell cycle, senescence, apoptosis, and metabolism by interacting with a number of molecules, including p53, promyelocytic leukemia protein, and peroxisome proliferators-activated receptor γ (4–6). Sirt1 has been recognized as a key regulator of vascular endothelial homeostasis controlling

From the Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan. Supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Culture, and Sports of Japan (20249041, 18590801, 18890056).

Manuscript received October 19, 2008; revised manuscript received December 17, 2008, accepted January 27, 2009.

angiogenesis, endothelial senescence, and dysfunction (7–9). Recently, we reported that an antiplatelet drug, cilostazol, inhibited oxidative stress-induced endothelial senescence through up-regulation of Sirt1 (10).

The aims of this study were to compare the effects of paclitaxel, sirolimus, and everolimus on the senescent phenotype in human endothelial cells and to further investigate the possible involvement of Sirt1 down-regulation as a mechanism. Potential protective effects of cilostazol and other widely used antiplatelet drugs (aspirin, ticlopidine, and clopidogrel) were also investigated.

Methods

Materials. Sirolimus and resveratrol were purchased from Wako Chemical Industries, Ltd. (Tokyo, Japan). Aspirin, ticlopidine, clopidogrel, paclitaxel, and everolimus were from Sigma (St. Louis, Missouri). 8-hydroxydeoxyguanosine (8-OHdG, AB5830) was from Chemicon (Temecula, California). Cilostazol was provided by Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan).

Cell culture. Human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex (Walkersville, Maryland), and maintained in endothelial growth medium (EGM-2) (EGM-2 single-quots, Cambrex). Population doubling levels were calculated as described previously (11), and all experiments were performed at population doubling level of 8 to 9.

Transform from ticlopidine and clopidogrel into liver metabolites.

Human liver microsomes were purchased from BD Gentest (Woburn, Massachusetts). As described by Savi et al. (12), liver microsomes were adjusted to 0.75 mg protein/ml in 100 mmol/l potassium phosphate buffer, pH 7.4, containing 100 mmol/l potassium fluoride and 10 mmol/l glutathione. Ticlopidine and clopidogrel were added at a final concentration of 1 mmol/l, and the reaction was initiated with 1 mmol/l β -nicotinamide adenine dinucleotide phosphate. Incubation was carried out at 37°C under continuous stirring (100 rpm) and protection from light using a reciprocal incubator. After 60 min, the incubation medium was cooled to 4°C and centrifuged at 10,000 g for 10 min, and supernatants were collected.

Senescence-associated β -galactosidase (SA- β gal) assay. The HUVEC were grown in 100-mm collagen-coated dishes to 80% confluence. They were pre-treated with vehicle (0.05% dimethylsulfoxide), cilostazol (10, 30, 100

Abbreviations and Acronyms

DES	= drug-eluting stent(s)
EGM	= endothelial growth medium
eNOS	= endothelial nitric oxide synthase
HUVEC	= human umbilical vein endothelial cells
PAI	= plasminogen activator inhibitor
SA-βgal	= senescence-associated β -galactosidase assay
SES	= sirolimus-eluting stent(s)
Sirt1	= silent mating type information regulation 2 homolog 1
ST	= stent thrombosis

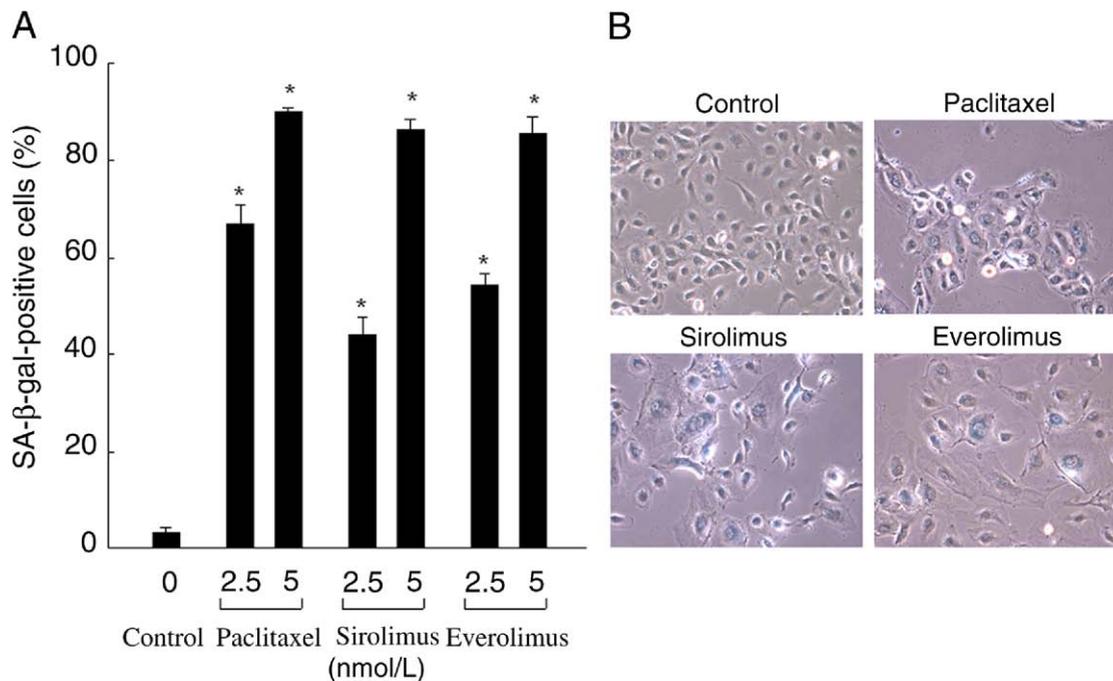


Figure 1. Paclitaxel, Sirolimus, and Everolimus Induced a Premature Senescent Phenotype

Paclitaxel, sirolimus, and everolimus (2.5, 5 nmol/l) induced a premature senescent phenotype in HUVEC as judged by SA- β gal (A) (unpaired t test, *p < 0.05 vs. control, n = 3) and morphological changes (B). HUVEC = human umbilical vein endothelial cells; SA- β gal = senescence-associated β -galactosidase assay.

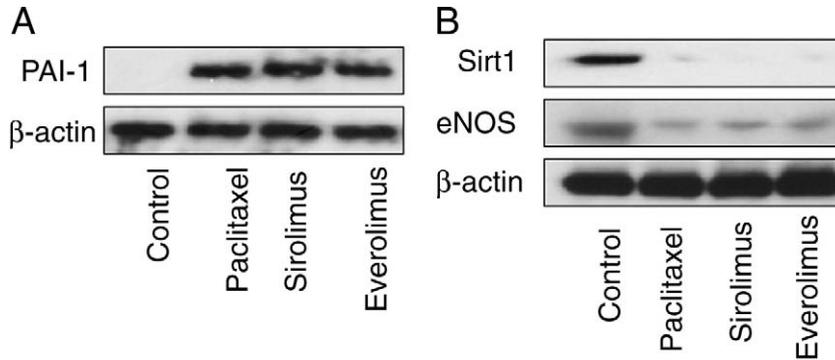


Figure 2 Expression of PAI-1, eNOS, and Sirt1 After Treatment With Paclitaxel, Sirolimus, or Everolimus

Expression of PAI-1 (A), eNOS, and Sirt1 (B) at 10 days after treatment with paclitaxel, sirolimus, or everolimus (2.5 nmol/l) were analyzed by Western blotting. Whole-cell lysates (20 μg) were prepared from treated HUVEC. Similar results were observed in 3 independent experiments. eNOS = endothelial nitric oxide synthase; PAI-1 = plasminogen activator inhibitor-1; Sirt1 = silent mating type information regulation 2 homolog 1.

μmol/l), aspirin (10, 30, 100 μmol/l), ticlopidine (10, 30, 100 μmol/l), clopidogrel (10, 30, 100 μmol/l), or resveratrol (10, 30, 100 μmol/l) diluted in EGM-2 medium for 24 h. Nitric oxide production was induced with calcium A 23187

ionophore (Sigma, 1 μmol/l) followed under incubation with aspirin, cilostazol, ticlopidine, or clopidogrel for 20 min (n = 3 samples in each experiment). The HUVEC were washed 3 times with EGM-2 and then treated for 24 h

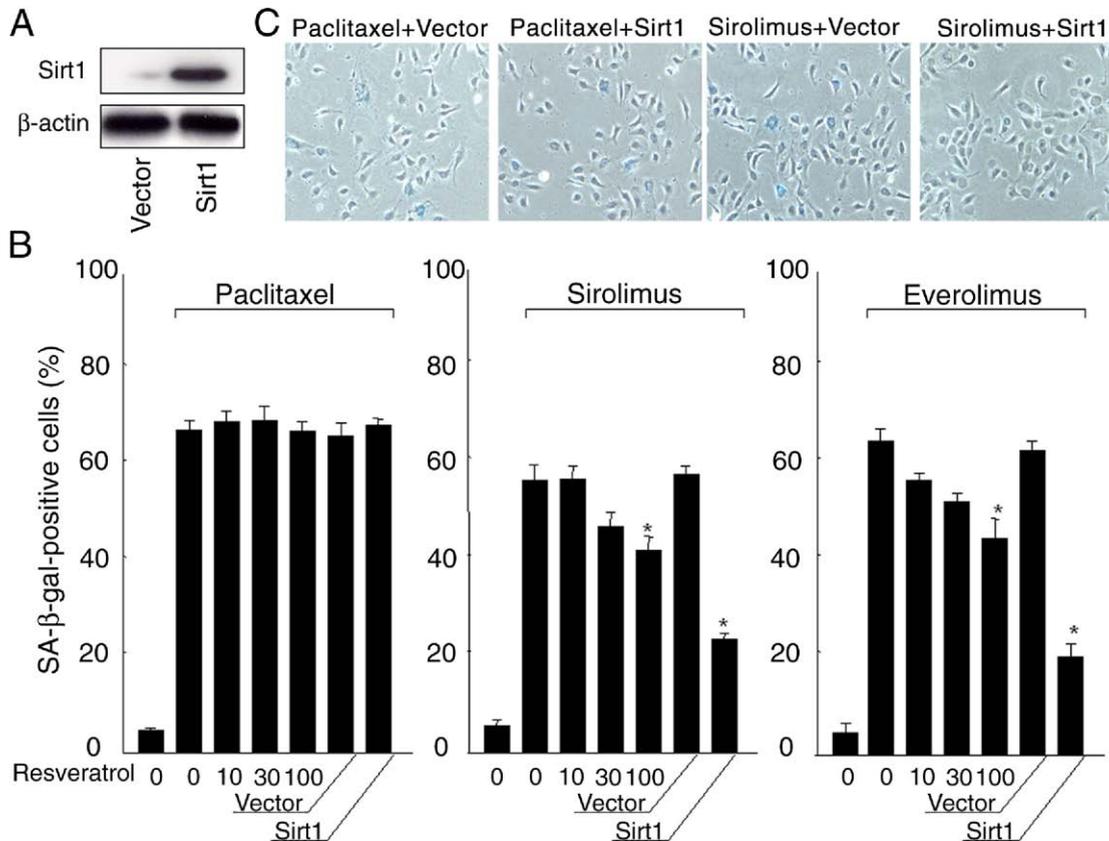


Figure 3 Overexpression of Sirt1 Inhibited Sirolimus- and Everolimus-Induced Senescent Phenotype

Overexpression of Sirt1 (A) or treatment with resveratrol (10, 30, 100 μmol/l) inhibited sirolimus- and everolimus-induced senescent phenotype as judged by SA-βgal (B) (unpaired t test, *p < 0.05 vs. control, n = 3) and morphological changes (C). Abbreviations as in Figures 1 and 2.

with paclitaxel, sirolimus, or everolimus (5 nmol/l) diluted in EGM-2. After the treatment, HUVEC were trypsinized, reseeded at a density of 1×10^5 in 60-mm dishes, and cultured in EGM-2 containing these compounds (cilostazol, aspirin, ticlopidine, or clopidogrel) for 10 days. At 10 days after treatment with paclitaxel, sirolimus, or everolimus, HUVEC were fixed and the proportion of SA- β gal positive cells was determined as described by Dimri *et al.* (13).

Sirt1 overexpression. The Sirt1 was overexpressed by transfection with pIRES-Sirt1 (10 μ g), which was provided by Dr. R. A. Weinberg (4,14), using jetPEI-HUVEC (Polyplustransfection, Illkirch, France) according to the manufacturer's instructions. At 3 days after transfection, HUVEC were treated with sirolimus, everolimus, or paclitaxel (2.5 nmol/l) for 24 h, washed 3 times with medium, and cultured for up to 10 days.

Immunoblotting. Cells were lysed on ice for 1 h in buffer (50 mmol/l Tris-HCl, pH 7.6, 150 mmol/l NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate [SDS], 1 mmol/l dithiothreitol, 1 mmol/l sodium vanadate, 1 mmol/l phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 10 mmol/l sodium fluoride). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking, the filters were incubated with the following antibodies; anti-endothelial nitric oxide synthase (eNOS) (BD Transduction Laboratories, Franklin Lakes, New Jersey), anti-Sirt1 (Santa Cruz Biotechnology, Inc., Santa Cruz, California), anti-plasminogen activator inhibitor-1 (PAI-1) (Molecular Innovations, Inc.) and anti- β -actin (Sigma). After washing and incubation with horseradish

peroxidase-conjugated antirabbit or antimouse immunoglobulin G (Amersham, Piscataway, New Jersey) for 1 h, the antigen-antibody complexes were visualized using an enhanced chemiluminescence system (Amersham).

Data analysis. Values are shown as mean \pm SEM in the text and figures. Unpaired Student *t* test was used for statistical analysis. Probability values <0.05 were considered significant.

Results

Paclitaxel, sirolimus, and everolimus induce premature senescence in human endothelial cells. To investigate the effect of paclitaxel, sirolimus, and everolimus on the endothelial senescent phenotype, we treated HUVEC with each drug for 24 h. Treatment with paclitaxel, sirolimus, and everolimus significantly induced a senescent phenotype as judged by SA- β gal and an enlarged, flattened cell morphological appearance at 10 days (Figs. 1A and 1B). Of note, paclitaxel had a tendency to arrest senescent growth, associated with round colony formation, because it may have the ability to stabilize microtubules and inhibit cell division.

Effects of paclitaxel, sirolimus, and everolimus on PAI-1, eNOS, and Sirt1 expression in human endothelial cells. Plasminogen activator inhibitor-1 is a potent inhibitor of fibrinolysis and a mediator of thrombosis, and its expression can also be used as a marker of endothelial senescence (15). In parallel with SA- β gal, expression of PAI-1 was increased by treatment with paclitaxel, sirolimus, and everolimus (Fig. 2A). Both eNOS and Sirt1 play a pivotal role in endothelial function and senescence (8–16); therefore, we examined

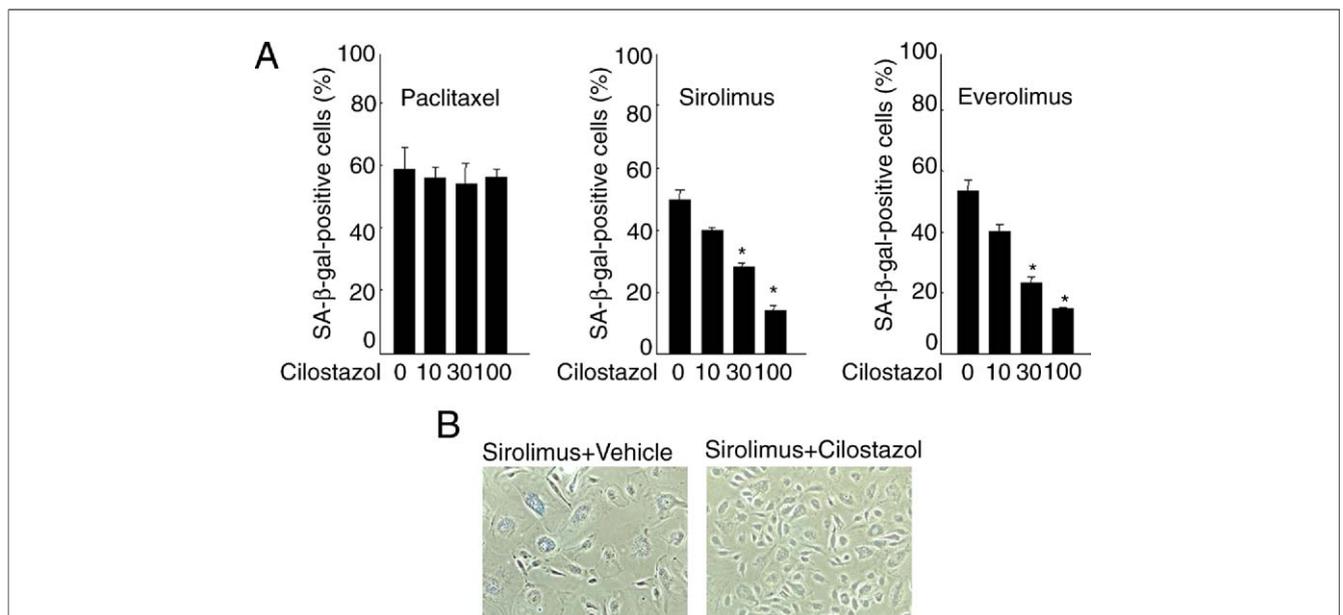


Figure 4 Effect of Cilostazol on Paclitaxel-, Sirolimus-, or Everolimus-Induced Senescent Phenotype

Effect of cilostazol (10, 30, 100 μ mol/l) on paclitaxel-, sirolimus-, or everolimus (2.5 nmol/l)-induced senescent phenotype as judged by SA- β gal (**A**) (unpaired *t* test, **p* < 0.05 vs. control, *n* = 3) and morphological changes (sirolimus) (**B**). Abbreviations as in Figure 1.

their expression. As shown in Figure 2B, paclitaxel, sirolimus, and everolimus decreased the expression of eNOS and Sirt1.

Overexpression of Sirt1 and resveratrol inhibited sirolimus- and everolimus-induced senescent phenotype.

To clarify the molecular mechanisms of regulation of endothelial senescence induced by paclitaxel, sirolimus, and everolimus, we induced Sirt1 gene overexpression and performed treatment with resveratrol, a Sirt1 chemical activator. We confirmed overexpression of Sirt1 at the protein level (Fig. 3A). As shown in Figures 3B and 3C, overexpression of Sirt1 and resveratrol decreased SA- β gal-positive cells and specific morphological senescent changes induced by sirolimus and everolimus. Unexpectedly, paclitaxel-treated cells could not be restored from a senescent phenotype. These results indicate that Sirt1 is a key molecule in the modulation of sirolimus- and everolimus- but not paclitaxel-induced senescence of HUVEC.

Cilostazol inhibits sirolimus- or everolimus-induced premature senescence. We examined the effect of cilostazol on a senescent phenotype in human endothelial cells. We pre-treated HUVEC with cilostazol for 24 h before the addition of paclitaxel, sirolimus, or everolimus. Treatment with cilostazol inhibited the senescent phenotype induced by sirolimus or everolimus at 10 days (Fig. 4A). Under treatment with sirolimus or everolimus (2.5 nmol/l), 49.2% or 53.0% of cells were SA- β gal positive, versus only 13.6%

or 14.6% of cilostazol (100 μ mol/l)-treated cells under the same conditions. Treatment with cilostazol decreased the specific senescent morphological changes and expression of PAI-1 induced by sirolimus and everolimus as well (Figs. 4B and 5A). Human umbilical vein endothelial cells treated with paclitaxel did not show a reduction of the senescent phenotype by treatment with cilostazol (Figs. 4A and 5A). At the same time, we checked the effect of other antiplatelet drugs (aspirin, ticlopidine, and clopidogrel) on the senescent phenotype (Figs. 5A and 6). Treatment with aspirin decreased SA- β gal-positive cells and expression of PAI-1, but treatment with ticlopidine or clopidogrel did not have any effect on them. To evaluate potential clinical interactions of ticlopidine and clopidogrel on endothelial senescence, we next used these compounds after incubation with human hepatic microsomes. Treatment with metabolites of ticlopidine and clopidogrel did not decrease the paclitaxel-, sirolimus-, or everolimus-induced senescent phenotype (paclitaxel, sirolimus, everolimus [2.5 nmol/l]; 61.4%, 55.7%, or 53.5% SA- β gal positive vs. 64.9%/62.2%, 55.9%/54.6%, or 55.8%/54.7% with metabolites of ticlopidine/clopidogrel [100 μ mol/l]).

Treatment with cilostazol reversed eNOS and Sirt1 expression. To explore the mechanism by which cilostazol prevented premature endothelial senescence induced by sirolimus and everolimus, we examined whether they could promote eNOS and the longevity gene, Sirt1. Cilostazol and aspirin

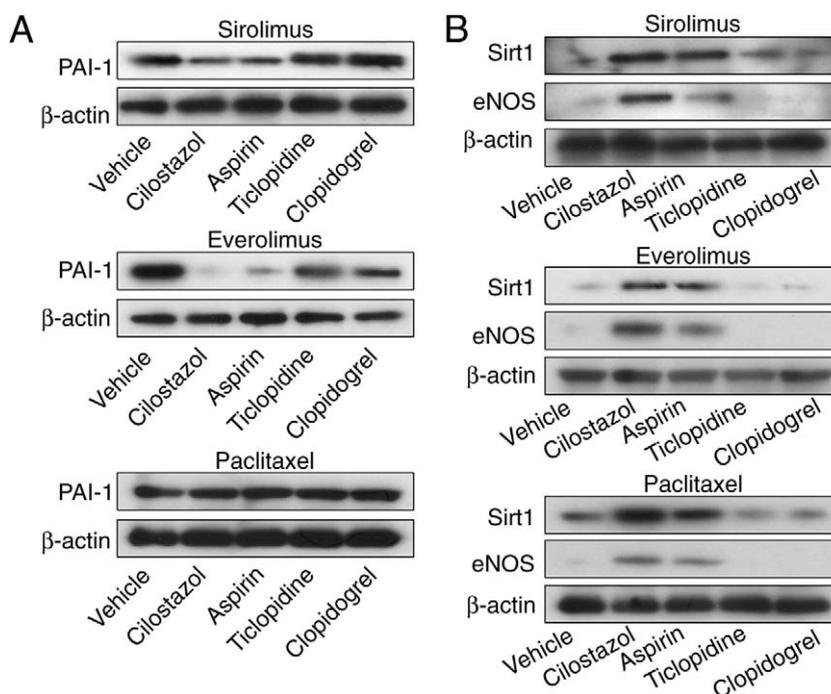


Figure 5 Expression of PAI-1, eNOS, and Sirt1 When HUVEC Were Treated With Cilostazol, Aspirin, Ticlopidine, or Clopidogrel

Expression of PAI-1 (A), eNOS, and Sirt1 protein (B) at 10 days when HUVEC were treated with cilostazol, aspirin, ticlopidine, or clopidogrel after addition of sirolimus, everolimus, or paclitaxel (2.5 nmol/l). Whole-cell lysates (20 μ g) were prepared from treated HUVEC. Similar results were observed in 3 independent experiments. Abbreviations as in Figures 1 and 2.

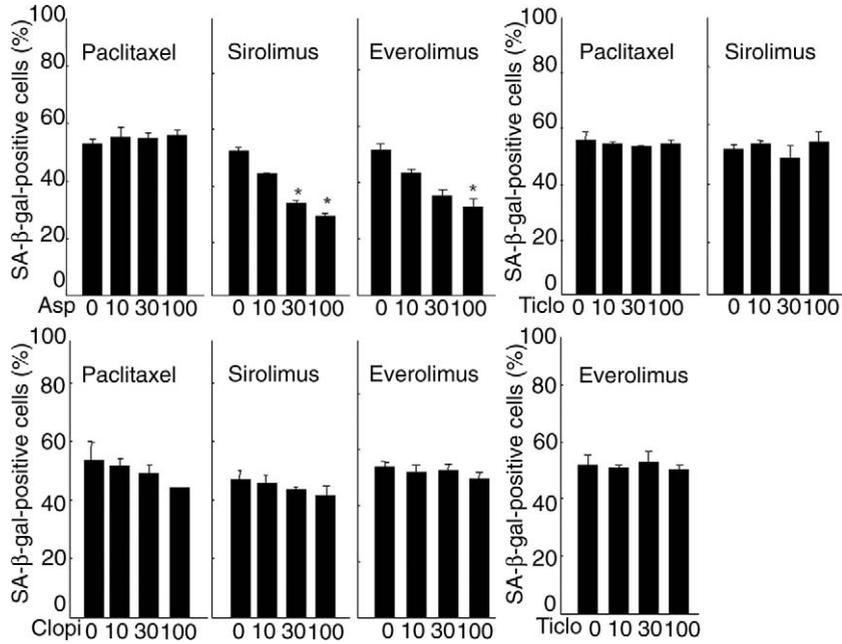


Figure 6 Effect of Aspirin, Ticlopidine, and Clopidogrel on Paclitaxel-, Sirolimus-, or Everolimus-Induced Senescent Phenotype

Effect of aspirin (asp), ticlopidine (ticlo), and clopidogrel (clopi) (10, 30, 100 μmol/l) on paclitaxel-, sirolimus-, or everolimus (2.5 nmol/l)-induced senescent phenotype as judged by SA-βgal (unpaired *t* test, **p* < 0.05 vs. control, *n* = 3). Abbreviations as in Figure 1.

significantly increased eNOS and Sirt1 protein at 10 days after treatment with sirolimus or everolimus (Fig. 5B). Although cilostazol and aspirin did not inhibit a paclitaxel-induced senescent phenotype, it reversed eNOS and Sirt1 expression (Fig. 5B). Moreover, treatment with cilostazol or aspirin decreased 8-OHdG, a marker of oxidative stress (Fig. 7).

Discussion

In the present study, we found that paclitaxel, sirolimus, and everolimus significantly induced endothelial senescence, with decreased eNOS and Sirt1 expression. Endothelial cell

senescence induced by sirolimus or everolimus was partly blunted by Sirt1 overexpression or Sirt1 activation by resveratrol, whereas senescence induced by paclitaxel occurred through a Sirt1-independent pathway.

Drug-eluting stents significantly reduce the rate of restenosis, but several pre-clinical and clinical safety concerns (17,18) related to its use have been expressed. One of the most important issues raised is ST, which is partly caused by delayed re-endothelialization. However, the pathogenesis of re-endothelialization or ST has not yet been fully explored (19), and evaluation of the degree

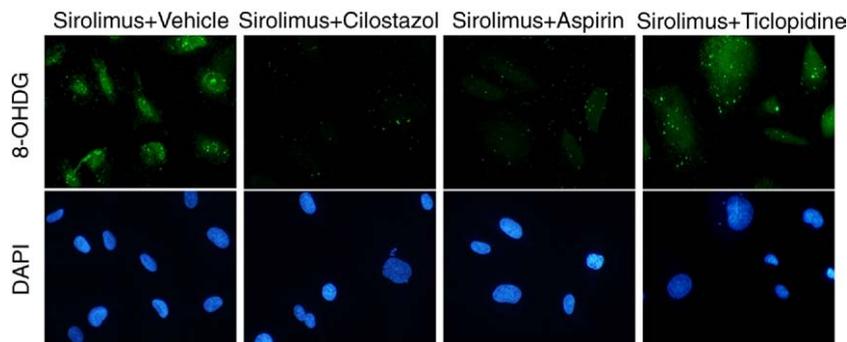


Figure 7 Antioxidative Effect of Cilostazol, Aspirin, Ticlopidine, and Clopidogrel on Sirolimus-Induced Senescent Phenotype

Effect of cilostazol, aspirin, ticlopidine, and clopidogrel (10, 30, 100 μmol/l) on sirolimus (2.5 nmol/l)-induced senescent phenotype as judged by immunofluorescent cell staining for 8-hydroxydeoxyguanosine (8-OHDG) (green) and diamidino-2-phenylindole dihydrochloride (DAPI) (nuclear staining, blue).

of endothelial senescence induced by drugs used for DES is of great significance.

First, we showed that paclitaxel, sirolimus, and everolimus significantly induced endothelial senescence, in association with reduced eNOS and increased PAI-1 expression, which are implicated in endothelial dysfunction and thrombogenesis. Similar to our results, recent studies showed that sirolimus increased tissue factor expression (1,20), and that both sirolimus and paclitaxel selectively increased PAI-1 expression (21). Secondly, we showed that overexpression of Sirt1 reversed the sirolimus- or everolimus-induced senescent phenotype. Interestingly, paclitaxel-induced senescence was not suppressed by Sirt1 overexpression. These results suggest that paclitaxel, sirolimus, and everolimus induce endothelial senescence through 2 distinct pathways, and sirolimus- and everolimus-induced senescence is mainly regulated by Sirt1. In addition to these findings, treatment with cilostazol reversed the senescent phenotype induced by sirolimus or everolimus, but not paclitaxel. As previously reported, cilostazol activates protein kinase A and PI3K/Akt signaling and increases eNOS expression (22). Recently, we reported that cilostazol inhibited oxidative stress-induced premature senescence via up-regulation of Sirt1 in human endothelial cells (10). These results imply that nitric oxide production by cilostazol increases Sirt1 expression, and increased Sirt1 expression inhibits endothelial senescence. Therefore, the eNOS–Sirt1 axis may play an important role in the modulation of sirolimus- or everolimus-induced premature senescence. Although cilostazol reversed eNOS and Sirt1 expression in HUVEC treated with paclitaxel, cilostazol did not rescue HUVEC from a senescent phenotype. Paclitaxel inhibits cell proliferation by promoting polymerization of tubulin dimers and subsequently stabilizing microtubules into an assembled state, which blocks the cell cycle from G2 to M phase. In contrast, sirolimus and everolimus bind to FKBP12 and subsequently bind to the mammalian target of rapamycin. This complex then increases cellular cyclin-dependent kinase inhibitor p27 and decreases the phosphorylation of retinoblastoma protein, which blocks the cell cycle from G1 to S phase (23). As previously reported (24), microtubules are not substrates of Sirt1. Because paclitaxel strongly stabilizes microtubules and inhibits cell division, we speculate that increased Sirt1 expression was insufficient for reversal of the paclitaxel-induced senescent phenotype, or there might be other regulators.

Resveratrol exerted a significant effect at a concentration of 100 $\mu\text{mol/l}$ (Fig. 3), which seems a relatively high concentration to achieve in vivo. However, considering that novel small molecule activators of Sirt1, even 1,000-fold more potent than resveratrol, are being identified (Sirtris Pharmaceuticals Inc., Boston, Massachusetts) (25), we suggest that using these chemical agents to activate Sirt1 might be of potential therapeutic value for protection against endothelial senescence.

In the current study, the impact of cilostazol in reducing post-operative sequelae seemed more marked in sirolimus-eluting stents (SES) than in paclitaxel-eluting stents. Moreover, SES plus triple treatment reduced restenosis more than did SES plus standard dual treatment (26). Cilostazol not only is an antiplatelet drug, but also has pleiotropic effects, including a vasodilating action, endothelial cell protection, and improvement of lipid metabolism (27–29). However, the impact of cilostazol on endothelial senescence after DES implantation has not been reported. Based on this study, we emphasize that cilostazol might have favorable effects against endothelial senescence when administered with sirolimus or everolimus, through up-regulation of Sirt1. To assess the implications of our findings, further studies are needed to examine the degree of senescence as well as the expression pattern of Sirt1 in the arterial wall after deployment of DES in vivo.

Conclusions

Our study shows that paclitaxel, sirolimus, and everolimus induce endothelial senescence through 2 distinct pathways. The development of endothelial senescence induced by sirolimus and everolimus is Sirt1-dependent, whereas paclitaxel acts through a Sirt1-independent pathway. Because sirolimus and everolimus (limus family) were involved in Sirt1 modulation, cilostazol reversed sirolimus- or everolimus-induced senescence. Our results may have an interesting clinical implication that triple antiplatelet therapy has more beneficial effects on endothelial senescence than has standard dual therapy of SES and everolimus-eluting stents.

Acknowledgments

The authors thank Dr. R. A. Weinberg (Member, Whitehead Institute for Biomedical Research Professor of Biology, MIT, Boston, Massachusetts) and Dr. M. Takata (Department of Immunology and Molecular Genetics, Kawasaki Medical School, Okayama, Japan) for providing the pIRES-Sirt1 plasmid.

Reprint requests and correspondence: Dr. Yasuyoshi Ouchi, Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: youchi-tky@umin.ac.jp.

REFERENCES

1. Lüscher TF, Steffel J, Eberli FR, et al. Drug-eluting stent and coronary thrombosis: biological mechanisms and clinical implications. *Circulation* 2007;115:1051–8.
2. Joner M, Finn AV, Farb A, et al. Pathology of drug-eluting stents in humans: delayed healing and late thrombotic risk. *J Am Coll Cardiol* 2006;48:193–202.
3. Minamino T, Miyauci H, Yoshida T, Ishida Y, Yoshida H, Komuro I. Endothelial cell senescence in human atherosclerosis: role of telomere in endothelial dysfunction. *Circulation* 2002;105:1541–4.
4. Vaziri H, Dessain SK, Ng Eaton E, et al. hSIR2 (SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* 2001;107:149–59.

5. Langley E, Pearson M, Faretta M, et al. Human SIRT2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence. *EMBO J* 2002;21:2383–96.
6. Picard F, Kurtev M, Chung N, et al. Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. *Nature* 2004;429:771–6.
7. Potente M, Ghaeni L, Baldessari D, et al. SIRT1 controls endothelial angiogenic functions during vascular growth. *Genes Dev* 2007;21:2644–58.
8. Ota H, Akishita M, Eto M, Iijima K, Kaneki M, Ouchi Y. Sirt1 modulates premature senescence-like phenotype in human endothelial cells. *J Mol Cell Cardiol* 2007;43:571–9.
9. Potente M, Dimmeler S. Emerging roles of SIRT1 in vascular endothelial homeostasis. *Cell Cycle* 2008;7:2117–22.
10. Ota H, Eto M, Kano MR, et al. Cilostazol inhibits oxidative stress-induced premature senescence via up-regulation of Sirt1 in human endothelial cells. *Arterioscler Thromb Vasc Biol* 2008;28:1634–9.
11. Maciag T, Hoover GA, Stemerman MB, Weinstein R. Serial propagation of human endothelial cells in vitro. *J Cell Biol* 1981;91:420–6.
12. Savi P, Pereillo JM, Uzabiaga MF, et al. Identification and biological activity of the active metabolite of clopidogrel. *Thromb Haemost* 2000;84:891–6.
13. Dimri GP, Lee X, Basile G, et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A* 1995;92:9363–7.
14. Matsushita N, Takami Y, Kimura M, et al. Role of NAD-dependent deacetylases SIRT1 and SIRT2 in radiation and cisplatin-induced cell death in vertebrate cells. *Genes Cells* 2005;10:321–32.
15. Comi P, Chiamonte R, Maier JA. Senescence-dependent regulation of type 1 plasminogen activator inhibitor in human vascular endothelial cells. *Exp Cell Res* 1995;219:304–8.
16. Hayashi T, Matsui-Hirai H, Miyazaki-Akita A, et al. Endothelial cellular senescence is inhibited by nitric oxide: implications in atherosclerosis associated with menopause and diabetes. *Proc Natl Acad Sci U S A* 2006;103:17018–23.
17. Farb A, Sangiorgi G, Carter AJ, et al. Pathology of acute and chronic coronary stenting in humans. *Circulation* 1999;99:44–52.
18. Virmani R, Guagliumi G, Farb A, et al. Localized hypersensitivity and late coronary thrombosis secondary to a sirolimus-eluting stent: should we be cautious? *Circulation* 2004;109:701–5.
19. Honda Y, Fitzgerald PJ. Stent thrombosis: an issue revisited in a changing world. *Circulation* 2003;108:2–5.
20. Steffel J, Latini RA, Akhmedov A, et al. Rapamycin, but not FK-506, increases endothelial tissue factor expression: implications for drug-eluting stent design. *Circulation* 2005;112:2002–11.
21. Muldowney JA 3rd, Stringham JR, Levy SE, et al. Antiproliferative agents alter vascular plasminogen activator inhibitor-1 expression: a potential prothrombotic mechanism of drug-eluting stents. *Arterioscler Thromb Vasc Biol* 2007;27:400–6.
22. Hashimoto A, Miyakoda G, Hirose Y, Mori T. Activation of endothelial nitric oxide synthase by cilostazol via a cAMP/protein kinase A- and phosphatidylinositol 3-kinase/Akt-dependent mechanism. *Atherosclerosis* 2006;189:350–7.
23. Daemen J, Serruys PW. Drug-eluting stent update 2007: part II: unsettled issues. *Circulation* 2007;116:316–28.
24. North BJ, Marshall BL, Borra MT, Denu JM, Verdin E. The human Sir2 ortholog, SIRT2, is an NAD⁺-dependent tubulin deacetylase. *Mol Cell* 2003;11:437–44.
25. Milne JC, Lambert PD, Schenk S, et al. Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. *Nature* 2007;450:712–6.
26. Lee SW, Park SW, Kim YH, et al. Drug-eluting stenting followed by cilostazol treatment reduces late restenosis in patients with diabetes mellitus the DECLARE-DIABETES trial (A randomized comparison of triple antiplatelet therapy with dual antiplatelet therapy after drug-eluting stent implantation in diabetic patients). *J Am Coll Cardiol* 2008;51:1181–7.
27. Tanaka K, Gotoh F, Fukuuchi Y, et al. Effects of a selective inhibitor of cyclic AMP phosphodiesterase on the pial microcirculation in feline cerebral ischemia. *Stroke* 1989;20:668–73.
28. Kim KY, Shin HK, Choi JM, Hong KW. Inhibition of lipopolysaccharide-induced apoptosis by cilostazol in human umbilical vein endothelial cells. *J Pharmacol Exp Ther* 2002;300:709–15.
29. Tani T, Uehara K, Sudo T, Marukawa K, Yasuda Y, Kimura Y. Cilostazol, a selective type III phosphodiesterase inhibitor, decreases triglyceride and increases HDL cholesterol levels by increasing lipoprotein lipase activity in rats. *Atherosclerosis* 2000;152:299–305.

Key Words: sirolimus ■ everolimus ■ antiplatelet therapy ■ endothelial senescence.