Inhibition of Intimal Thickening After Balloon Angioplasty in Porcine Coronary Arteries by Targeting Regulators of the Cell Cycle

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Background—Although percutaneous transluminal coronary angioplasty (PTCA) is a highly effective procedure to reduce the severity of stenotic coronary atherosclerotic disease, its long-term success is significantly limited by the high rate of restenosis. Several cellular and molecular mechanisms have been implicated in the development of restenosis post-PTCA, including vascular smooth muscle cell (VSMC) activation, migration, and proliferation. Recently, our group demonstrated that rapamycin, an immunosuppressant agent with antiproliferative properties, inhibits both rat and human VSMC proliferation and migration in vitro. In the present study, we investigated (1) whether rapamycin administration could reduce neointimal thickening in a porcine model of restenosis post-PTCA and (2) the mechanism by which rapamycin inhibits VSMCs in vivo.

Methods and Results—PTCA was performed on a porcine model at a balloon/vessel ratio of 1.7 ± 0.2. Coronary arteries were analyzed for neointimal formation 4 weeks after PTCA. Intramuscular administration of rapamycin started 3 days before PTCA at a dose of 0.5 mg/kg and continued for 14 days at a dose of 0.25 mg/kg. Cyclin-dependent kinase inhibitor (CDKI) p27kip1 protein levels and pRb phosphorylation within the vessel wall were determined by immunoblot analysis. PTCA in the control group was associated with the development of significant luminal stenosis 4 weeks after the coronary intervention. Luminal narrowing was a consequence of significant neointimal formation in the injured areas. Rapamycin administration was associated with a significant inhibition in coronary stenosis (63 ± 3.4% versus 36 ± 4.5%; P < 0.001), resulting in a concomitant increase in luminal area (1.74 ± 0.1 mm² versus 3.3 ± 0.4 mm²; P < 0.001) after PTCA. Inhibition of proliferation was associated with markedly increased concentrations of the p27kip1 levels and inhibition of pRb phosphorylation within the vessel wall.

Conclusions—Rapamycin administration significantly reduced the arterial proliferative response after PTCA in the pig by increasing the level of the CDKI p27kip1 and inhibition of the pRb phosphorylation within the vessel wall. Therefore, pharmacological interventions that elevate CDKI in the vessel wall and target cyclin-dependent kinase activity may have a therapeutic role in the treatment of restenosis after angioplasty in humans. (Circulation. 1999;99:2164-2170.)

Key Words: restenosis ▶ cells ▶ angioplasty

vascular smooth muscle cell (VSMC) proliferation and migration contribute significantly to the restenotic process after percutaneous transluminal coronary angioplasty (PTCA) and accelerated arteriosclerosis after cardiac transplantation. Long-term success is limited by the high rate of restenosis, which affects ≈40% to 50% of the patients undergoing PTCA.1–3 Pathological, clinical, and experimental evidence suggests that multiple cellular and molecular mechanisms are involved in a cascade of events that lead to restenosis. Autocrine and paracrine mediators triggered by the coronary intervention induce arterial narrowing as a result of the increased VSMC proliferation and synthesis of extracellular matrix.4–6 Attention has been focused on elucidating the mechanisms underlying VSMC proliferation with the goal of developing therapeutic approaches to inhibit restenosis after PTCA.7–9

Arterial injury during PTCA induces multiple signaling pathways that activate VSMC migration and proliferation. Immediately after injury, VSMCs leave their quiescent state and enter the cell cycle, associated with the induction of early-response genes.10–12 Cell division and growth are tightly controlled by a series of positive and negative regu-
lators that act at sequential points throughout the cell cycle. Studies show that antisense oligonucleotides targeted to cyclin-dependent kinases (cdc2 and cdk2) can be effective in inhibiting VSMC proliferation and restenosis. Currently, our group and others identified the tumor suppressor protein retinoblastoma protein (pRb) as a critical regulator of cell-cycle transition and proliferation. The inhibition of pRb phosphorylation by either antisense oligonucleotides to cyclin-dependent kinases (CDK) or pharmacological agents such as rapamycin results in cell-cycle arrest in VSMCs and inhibition of proliferation.

The kinase activity of the cyclin/CDK complex is regulated by their interaction with inhibitors (CDKI), such as p16, p21, and p27kip1. CDKIs act stoichiometrically, and oscillations in their levels profoundly impact cell-cycle progression. In a rat carotid model of balloon angioplasty, adenovirus-mediated overexpression of the CDKI p21 inhibited pRb phosphorylation and VSMC proliferation. Defective regulation of p27kip1, either secondary to constitutively low levels of p27kip1 or due to a targeted disruption of the p27kip1 gene (p27 knockout mouse), results in rapamycin resistance.

Recently, Marx et al reported that rapamycin, a macrolide antibiotic, inhibited both human and rat VSMC proliferation in vitro by blocking G1/S transition. The inhibition of proliferation was mediated by rapamycin binding to its cytosolic receptor, FKBP12, and associated with reduced cdc2 and cdk2 activity and pRb phosphorylation. In addition, rapamycin inhibits rat, porcine, and human VSMC migration. Rapamycin also prevents the downregulation of p27kip1 in a myogenic cell line, BC3H1, thus contributing to cell-cycle arrest. The observation that rapamycin blocks VSMC proliferation and migration in vitro suggests that rapamycin could prevent VSMC activation after PTCA regardless of the mechanism(s) that initiates the process. Several studies suggested that rapamycin, but not FK506, retards the development of graft vessel disease after cardiac transplantation and restenosis after mechanical injury in rat. In the present study, we investigated whether rapamycin effectively interferes with the pathological proliferative response after coronary angioplasty in swine. Our results indicate that rapamycin reduced intimal thickening by 50% after coronary angioplasty. This effect is marked by a selective inhibition of the VSMC proliferative response to coronary injury associated with decreased pRb phosphorylation and elevated levels of p27kip1 causing cell-cycle arrest at G1/S. Thus, like adenovirus-mediated overexpression of a CDKI, we demonstrate that a pharmacological approach to elevate a CDKI in the coronary arteries results in the inhibition of restenosis after PTCA.

Methods

Animal Species

The study used Yorkshire-Albino swine (27 to 32 kg body weight). Interventions procedures and animal handling were approved by the Mount Sinai School of Medicine Animal Management Program, which is accredited by the American Association for the Accreditation of Laboratory Animal Care and meets NIH and AHA standards.

Experimental Design

Rapamycin (Sirolimus) was a gift from Wyeth-Ayerst Research Laboratory (Dr Suren Sehgal). Despite its oral bioavailability, rapamycin was administered intramuscularly to ensure stable blood levels. Rapamycin administration was started 3 days before angioplasty at a dose of 0.5 mg/kg and continued for 14 days at a dose of 0.25 mg/kg. Before administration, rapamycin was suspended in a sterile solution containing 0.2% sodium carboxymethyl cellulose and 0.25% polysorbate-80. The control group received the vehicle solution.

Coronary balloon angioplasty was carried out in the pigs as previously reported. All pigs were anticoagulated with heparin (100 IU/kg) after insertion of the catheters. This anticoagulation regimen attained an activated partial thromboplastin time ratio 2 to 3 times control. PTCA was performed by 3 inflations at 8 to 10 atm of a 4-mm balloon for 15 seconds with 60-second rest periods between inflations. After the angioplasty procedure, the animals were allowed to recover, returned to their pens, and followed up for 28 days.

Fixation, Harvesting, and Pathological Evaluation of Injured Vessels

All 2-mm coronary segments were evaluated by 2 experienced observers blinded to the treatment group. For each angioimplanted coronary artery, the section with the most extensive injury-induced response was identified and evaluated as reported. A total of 17 coronary arteries from 10 animals in the rapamycin-treated group and 22 coronary arteries from 10 animals in the control group were studied.

The neointima was subdivided into 3 distinct areas that were clearly visible by direct histological examination. These 3 areas were defined as organizing thrombus, submedial hematoma, and fibrocellular hyperplasia. Organizing thrombus was defined as remnants of an original thrombus induced at the time of angioplasty and undergoing organization. It was usually observed beneath or within the proliferative lesion present between the breaks in the internal elastic lamina and media. Submedial hematoma was defined as a clot formed under medial flaps or dissections. Fibrocellular hyperplasia was defined as those areas composed solely of cellular elements and extracellular matrix.

Measurements of Retinoblastoma Protein and p27kip1 Levels

Rb phosphorylation and p27kip1 in the vessel wall were determined as previously described. Briefly, arterial tissues were homogenized with a tissue blender in a lysis buffer. Protein extracts (300 µg) were size-fractionated on 12% SDS-polyacrylamide gels, transferred to nitrocellulose overnight. Filters were incubated overnight with an affinity-purified polyclonal antibody to Rb (Pharmingen) and p27 (Santa Cruz Biotechnology; sc-528), respectively. Membranes were washed 4 times with Tris-buffered saline containing 0.1% Tween-20 and incubated with the secondary antibody (goat anti–mouse IgG for pRb and goat anti–rabbit IgG for p27) (1:1000) for 1 hour. The membranes were washed and the signals detected by the ECL chemiluminescence detection system (Amersham). Autoradiographic signals are quantified by densitometry.
Statistical Analysis
Data are presented as mean±SEM unless otherwise stated. Absolute areas are expressed as mm$^2$. The statistical significance of differences between the normal and treated groups was determined by a 1-way ANOVA. Differences were considered significant if $P<0.05$ by use of StatView 512+ statistical software (Brain Power, Inc).

Results

Arterial Injury Induced by the Angioplasty Procedure
All coronary segments analyzed for intimal proliferation after angioplasty were histologically characterized by disruption of the internal elastic lamina, with laceration of the tunica media and exposure of the external elastic lamina. The degree of arterial injury induced by the coronary intervention was similar in both groups. The average balloon/vessel ratio was 1.5±0.2 versus 1.5±0.1 in the control and rapamycin-treated animals, respectively.

Intimal Proliferation After Arterial Injury
At death, 4 weeks after angioplasty, both groups of animals showed a similar coronary size, assessed by the vascular area encircled by the external elastic lamina (7.3±0.5 versus 7.6±0.4 mm$^2$ for the rapamycin and the control group, respectively). When the luminal area was evaluated, a statistically significantly larger lumen was observed in the rapamycin-treated animals compared with the controls (3.3±0.4 versus 1.74±0.1 mm$^2$, respectively; $P<0.0001$) (Figure 1A). The significant luminal narrowing in the control group resulted from a larger standardized intimal-medial ratio (4.0±0.8 controls versus 1.9±0.4 rapamycin; $P<0.001$) (Figure 1B), which indicated a reduced proliferative response to angioplasty-induced arterial injury in the treated group.

 Coronary Stenosis
Results of the average values of coronary stenosis induced by angioplasty 4 weeks after intervention are presented in Figure 2. Representative photomicrographs of histological sections from the control and rapamycin-treated groups are depicted in Figure 3. These figures show a significant intimal proliferation in the control arteries. The newly formed proliferative tissue, characterized by spindle-shaped cells, filled gaps between medial tears and generally extended to adjacent medial areas encroaching into the lumen. The administration of rapamycin was associated with a significant reduction in the percentage of luminal stenosis induced by the angioplasty (36±4.5% versus 63±3.4%, control versus rapamycin; $P<0.0001$). Total stenosis was subdivided into its 3 major components: residual thrombus, organizing hematoma, and fibrocellular hyperplasia. No statistically significant differences were observed in the percentage of either residual thrombus (12.3±2.3% versus 9.2±1.8%, controls versus rapamycin, respectively) or hematoma (6.6±1.5% versus 6.1±2.2%, controls versus rapamycin). In contrast, there was a marked reduction in the percentage of fibrocellular hyperplasia (43.8±2.2% versus 21.0±3.1%, controls versus rapamycin; $P<0.0001$). This finding indicates that the inhibitory effect of rapamycin on intimal proliferation after coronary angioplasty was mediated through specific inhibition of VSMC proliferation.

Vessel Wall p27kip1 Protein Determination
In various cell types, the decrease in p27 levels on addition of mitogens activates CDKs, leading to pRb hyperphosphorylation and cell-cycle progression and proliferation.\textsuperscript{32} To demonstrate that the effect of rapamycin on SMC proliferation was mediated by this mechanism, we examined the level of p27kip1 in the vessel wall of animals undergoing balloon angioplasty treated with rapamycin or vehicle. Administration of rapamycin resulted in a significant increase in p27kip1 at the selected time points of 3 and 15 days after PTCA (Figure 4). These time points were selected on the basis of previously reported data in different animal models suggesting that cell proliferation peaks within the first 3 days postintervention and that the 15-day point corresponded to the end of therapy administration. Therefore, rapamycin administration prevents the downregulation of the CDKI p27kip1 in the vessel wall post-PTCA in the pig. Thus, pharmacological manipulation of CDKI levels results in inhibition of VSMC proliferation.

Vessel Wall Retinoblastoma Protein Phosphorylation
We also analyzed the effects of rapamycin administration on the Rb phosphorylation in the coronary wall after angioplasty. Immunoblot analysis of pRb protein levels in coronary arteries of rapamycin-treated and control animals is presented in Figure 5. Figure 5 shows the inhibitory effects of rapamycin on the phosphorylation of the pRb, as indicated by the presence of a lower band corresponding to the underphosphorylated Rb (pRB). Conversely, the vehicle-treated coronary arteries showed 1 single band corresponding to the hyperphosphorylated pRb (ppRb). Our observations indicate that rapamycin also interferes with the phosphorylation of the Rb protein.

Rapamycin Blood Levels
A loading dose of 0.5 mg·kg$^{-1}$·d$^{-1}$ for 3 days before PTCA attained plasma levels of 57 ng/mL of blood at the time of the coronary intervention. The administration of a lower maintenance dose (0.25 mg·kg$^{-1}$·d$^{-1}$) maintained similar blood levels (59±12 ng/mL).
day 14 after PTCA. Interestingly, 14 days after the last rapamycin injection, the average level of rapamycin was 36±3 ng/mL of blood. These pharmacokinetic observations indicate that the biodistribution of this compound is associated with a depot effect when administered intramuscularly that might account for the prolonged blood residence time of this compound. No correlation was found between individual rapamycin blood levels and the corresponding values of intimal thickening, suggesting that the lower blood concentrations were as effective as the higher ones.

**Discussion**

This study demonstrates that rapamycin inhibits intimal proliferation after PTCA in the pig model. Our results show a 50% reduction in fibrocellular hyperplasia in the rapamycin-treated animals compared with controls. The observed antiproliferative effect of rapamycin is associated with an inhibition of the pRB phosphorylation and increase in CDKI p27kip1 levels. These findings suggest that rapamycin could serve as a new therapeutic approach to reduce human restenosis after PTCA. The use of a pharmacological agent specifically targeted to elevate the level of a CDKI within the vessel wall represents a novel approach in the prevention of restenosis.

Rapamycin, a macrolide antibiotic with antifungal and immunosuppressive activities, inhibits the development of arteriopathy after allograft transplantation. The drug is currently in phase II and III clinical trials in liver, kidney, and heart transplantation. Rapamycin exerts its antiproliferative activity after binding to the cytosolic protein FKBP12. The FKBP12-rapamycin complex is an inhibitor of a 289-kDa protein named FRAP or RAFT1, which is a member of a newly discovered family of phosphatidylinositol kinase–related kinases. Furthermore, rapamycin inhibits translation of a subset of mRNA that is believed to be critical for G1 progression. Increased p27 levels cause inhibition of CDK activity, which leads to the inhibition of pRb phosphorylation in vivo. The underphosphorylated form of pRb is believed to be a functionally active form of pRb in G0/mid G1. It is proposed that pRb forms complexes while underphosphorylated with DNA-binding proteins, such as E2F. On pRb phosphorylation, unbound E2F appears to stimulate transcription of cellular genes implicated in induction of the S phase. Thus, inhibition of pRb hyperphosphorylation by rapamycin interferes with cell-cycle signaling and cell proliferation.

Severe arterial injury in the swine model, whether induced by PTCA or stent implantation, triggers a hyperplastic re-
response characterized by the development of intimal thickening in the injured arterial segments. Similar results have been described by others. In all instances, the greatest proliferative response occurs at the sites of maximal injury (Figure 3). In our study, the severity of the arterial injury induced by the angioplasty procedure was similar in the 2 groups of animals, as shown by the damage index and the percentage of missing internal elastic lamina. Because there was no difference in the degree of arterial injury between control and rapamycin-treated animals, the observed reduction in luminal narrowing was a consequence of the treatment and the elevation of the CDKI p27kip1 in the vessel wall.

The proliferative response after PTCA observed in the control group was clearly manifested by the thickening of the intimal layer and a reduction in the coronary lumen as shown in Figures 2 and 3. The rapamycin-treated animals exhibited a significantly larger lumen compared with the controls, as well as a reduction in intimal thickening. Animals receiving rapamycin showed an ≈50% decrease in the fibrocellular response (41.2±2.3% controls versus 21.0±3.1% rapamycin animals; P<0.0001). No differences were observed in the contribution of residual thrombus or hematoma to total luminal stenosis. The significant inhibition in fibrocellular hyperplasia supports in vitro data demonstrating that rapamycin inhibits SMC proliferation and migration.

Administration of rapamycin was initiated 3 days before angioplasty to ensure that VSMCs would be maintained in a quiescent state from the moment of injury. Rapamycin administration was then maintained for an additional 14 days to prevent any growth stimulus that might occur during the subsequent 2 weeks after the procedure. This regimen attained significant blood levels even 14 days after the last rapamycin administration. This longer blood residence time might also be important to the observed inhibitory effect on neointimal formation after PTCA.

Figure 4. Effect of rapamycin on p27kip1 levels in coronary arteries. Rapamycin increased levels of p27kip1 compared with controls. These effects are observed both at 3 (left) and at 15 (right) days after PTCA. Note that effect of rapamycin in p27 levels is more marked in those coronaries undergoing PTCA. Results are expressed as arbitrary units (mean±SEM; n=4).
The antiproliferative activity of rapamycin in conjunction with its immunosuppressive properties suggests that this drug could also be useful for the prevention and/or treatment of accelerated arteriopathy associated with organ transplantation, particularly in the case of cardiac transplantation, in which accelerated arteriopathy is a major cause of mortality and morbidity and the need for repeat transplantation.

After arterial injury, multiple mitogenic and proliferative factors have been identified as capable of triggering signaling mechanisms leading to SMC activation. Numerous pharmacological agents, including antiplatelet agents, anticoagulants, ACE inhibitors, and cytotoxic agents, have not significantly reduced restenosis after angioplasty. However, because rapamycin inhibits multiple regulators of cell-cycle progression in VSMCs, its mechanism of action differs from many of these agents. Moreover, the ability of rapamycin to inhibit in vitro VSMC proliferation in rodents, humans, and now in vivo in pigs suggests that its efficacy, unlike many other antiproliferative agents that have failed in human trials, is not species-specific.

Numerous studies have reported beneficial effects of gene-based therapy to inhibit restenosis after PTCA. Delivery of antisense oligonucleotides to c-myb, cdc2, and PCNA or cdk2 have been reported to inhibit restenosis in the rat carotid artery model. However, there are several disadvantages with the use of antisense technology, including nonspecific effects, batch variability, and difficulty with the delivery system. In addition, adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene followed by administration of ganciclovir has been shown to inhibit restenosis in rat and pig. Several disadvantages of this approach include the induction of cell death, which may lead to intravascular inflammation and the potential for medial necrosis and aneurysm formation, as well as side effects of ganciclovir therapy.

Another approach used was the adenovirus-mediated overexpression of either a nonphosphorylatable constitutively active form of pRb or the overexpression of the CDKI p21. Another approach was used the adenovirus-mediated overexpression of either a nonphosphorylatable constitutively active form of pRb or the overexpression of the CDKI p21. Because rapamycin can be administered orally and causes the inhibition of pRb phosphorylation, as well as increased levels of the CDKI p27kip1 in the vessel wall, rapamycin may be an ideal agent to inhibit restenosis post-PTCA.

In summary, our study demonstrates that rapamycin significantly reduces the proliferative response after coronary angioplasty in the pig. Because rapamycin targets fundamental regulators of cell growth, its ability to inhibit experimental stenosis in the pig coronary model suggests that these regulators play a significant role in the pathogenesis of injury-induced intimal thickening. Moreover, these results suggest that administration of rapamycin to patients post-PTCA may have a role in the inhibition of restenosis.

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