Oral Everolimus Inhibits In-Stent Neointimal Growth
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Background—Rapamycin (sirolimus)-eluting stents are associated with reduced restenosis rates in animal studies and initial human trials. The present study evaluated whether orally administered everolimus (a macrolide of the same family as sirolimus) inhibits in-stent neointimal growth in rabbit iliac arteries.

Methods and Results—New Zealand white rabbits were randomized to everolimus 1.5 mg/kg per day starting 3 days before stenting and reduced to 1 mg/kg per day for 28 days (group 1), everolimus 1.5 mg/kg given 1 day before stenting followed by 0.75 mg/kg per day for 28 days (group 2), or matching placebo for each group. Drugs were administered by oral gavage. Stents were deployed in both iliac arteries, and arteries were harvested 28 days after stenting. Group 1 everolimus-treated rabbits experienced weight loss and anorexia, which resolved after the everolimus dose was lowered on day 14. Group 2 animals were healthy for the duration of everolimus dosing. Both everolimus treatment groups significantly reduced in-stent neointimal growth (46% reduction and 42% reduction in intimal thickness in groups 1 and 2, respectively). In group 2 everolimus-treated animals, the neointima was healed or healing, characterized by stent struts covered by a thin neointima, overlying endothelial cells, and only small foci of fibrin. Scanning electron microscopy showed >80% stent surface endothelialization in group 2 everolimus-treated rabbits.

Conclusions—Oral everolimus suppresses in-stent neointimal growth in the rabbit iliac artery. At a dose of 1.5 mg/kg given 1 day before stenting followed by 0.75 mg/kg per day for 28 days, everolimus was well tolerated and was associated with significant neointimal healing. (Circulation. 2002;106:2379-2384.)

Key Words: pathology ■ restenosis ■ stent

Rapamycin (sirolimus), a macrolide immunosuppressant inhibitor of mTOR (mammalian target of Rapamycin), inhibits growth factor–dependent proliferation of hematopoietic and nonhematopoietic cells (vascular smooth muscle cells and fibroblasts) via cell-cycle arrest in the late G1 phase. Rapamycin has been shown to inhibit vascular smooth muscle cell proliferation and migration in vitro and inhibit neointimal growth in balloon-injured rat carotid and porcine coronary arteries. More recently, sirolimus-coated stents were shown to inhibit in-stent neointimal growth in porcine coronary arteries at 28 days, and impressive initial results with sirolimus-eluting stents in humans (0% restenosis rate at 210 days) have been reported.

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Previous studies of systemic therapy (eg, lipid-lowering agents, antioxidants, and antithrombotic drugs) to prevent restenosis after balloon angioplasty have been largely disappointing. Antirestenosis therapy offered by drug-releasing stents has obvious merits in the ability to target therapy locally. However, animal studies of paclitaxel and sirolimus drug-eluting stents have also demonstrated delayed neointimal healing. Furthermore, because late neointimal catch-up remains a potential adverse outcome with stent-based drug delivery, there may arise a need for adjunctive systemic therapies to maintain neointimal inhibition. Pharmacokinetic studies of oral sirolimus have shown wide interindividual variability. Everolimus [40-O-(2-hydroxyethyl)-rapamycin], an orally active immunosuppressive and antiproliferative compound of the same family as sirolimus, has shown promise in preventing rejection in renal and heart transplantation. The present study was designed to test the hypothesis that everolimus can inhibit in-stent neointimal growth when given orally.

Methods
All test drugs were diluted in a cremophor-based vehicle and administered daily by oral gavage in a volume of 2 mL/kg. Two different dosing regimens were evaluated.

Group 1
Everolimus (1.5 mg/kg per day) was started 3 days before stenting and reduced to 1 mg/kg per day (secondary to weight loss) from days 14 to 28.
Group 2
Everolimus loading dose of 1.5 mg/kg was given 1 day before stenting followed by everolimus 0.75 mg/kg per day for 28 days. For each group, control animals were gavaged with a matching volume of vehicle (group 1 placebo and group 2 placebo). Rabbits were randomly assigned to each of the treatment groups. Everolimus was supplied by Novartis Pharmaceuticals Corporation. For the entire study, all animals were fed with Teklad Global High Fiber Rabbit Diet.

Stent Procedure and Tissue Harvest
Under fluoroscopic guidance, bilateral iliac artery balloon injury was performed in anesthetized rabbits followed by placement of a 3×12-mm MULTI-LINK (Guidant Corp) stent (6 ATM, 30-second balloon inflation, stent-to-artery ratio of 1.2:1). All animals received aspirin 40 mg/d orally until euthanasia. Twenty-eight days after stenting, animals were anesthetized, and a pre-euthanasia angiogram of the iliac arteries was completed followed by euthanasia and perfusion-fixation. The stented arteries were embedded in methylmethacrylate with sections taken from the proximal, middle, and distal portions of each stent. A 3-mm arterial segment just proximal and distal to the stents was processed and stained to evaluate edge effects. All sections were stained with H&E and Movat pentachrome stain. To assess cellular proliferation, animals received bromodeoxyuridine (BrdU) before euthanasia, as previously described.\(^17\) Mid-stent sections were also stained with antibodies to RAM11 (Dako Corp) to identify macrophages and fibrin (American Diagnostica, Inc). To evaluate stent endothelialization, additional rabbits underwent stenting, as described above, with either group 2 everolimus (n=4) or placebo (n=4) dosing for 28 days followed by scanning electron microscopy of longitudinally cut stents.

Data Analysis
All arterial segments were examined blindly. Computerized planimetry was performed on all stented sections, as previously described.\(^17\) Total cell number, RAM11-positive macrophages, and fibrin were quantitated from the entire neointima of midstent sections. The neointimal cell proliferation index (percent proliferating cells) was defined as the ratio of BrdU-positive neointimal cells to total neointimal cell number. The percent of the stent intimal surface that was endothelialized was measured from scanning electron microscopy images of the entire stent surface. Data are expressed as mean±SD. Incomplete neointimal healing was defined as the presence of fibrin or inflammation or the absence of endothelial cells. Statistical analysis of the histologic data was accomplished using ANOVA. \(P<0.05\) was considered statistically significant.

Results
In group 1 everolimus-treated animals, 5 of 6 experienced weight loss (≈10% body weight) and loss of appetite between 5 to 7 days after stent implant but appeared healthy otherwise. Thereafter, the animals appeared well and tolerated the reduced dose of everolimus (from 1.5 to 1 mg/kg per day on day 14 after stenting) without problems. All group 1 everolimus animals regained weight after the dose of everolimus was reduced so that by 28 days, 4 of 6 rabbits were within 0.1 kg of their initial weight, 1 rabbit was within 0.3 kg of its initial weight, and 1 rabbit was within 0.5 kg of its initial weight. All group 2 rabbits and all placebo animals (groups 1 and 2) appeared healthy, without weight loss for the duration of the protocol. The plasma everolimus level of group 2 rabbits measured 1 hour after gavage 28 days after stent implant was 62.6±6.6 ng/mL. All arteries were widely patent at follow-up angiography at 28 days.

Twelve stents deployed in 6 group 1 everolimus-treated rabbits were compared with 12 stents in 6 placebo-treated animals. Everolimus was associated with a significant reduction in neointimal thickness (46% reduction), neointimal area (35% reduction), and percent stent stenosis (36% reduction), with a 14% increase in lumen area (Table 1). The area within the internal elastic lamina was similar in everolimus and placebo-treated animals, indicating that everolimus was associated with neither positive nor negative arterial remodeling. However, there was evidence of medial thinning and hypocellularity in the everolimus group; the mean medial area was 0.29±0.01 mm\(^2\) in everolimus animals versus 0.42±0.01 mm\(^2\) in placebo animals (\(P<0.001\)).

The in-stent neointima of placebo animals at 28 days appeared fully healed, consisting of abundant smooth muscle cells in a proteoglycan/collagen matrix (Figures 1A and 1B). The extent of healing was variable in group 1 everolimus-treated animals. In some arterial segments, healing was characterized by a compact intimal covering over and between stent struts; in others, there was intima between struts with struts themselves uncovered by neointima or endothelium (Figures 1C through 1E). Rarely, both strut and interstrut regions had no overlying neointima. Focal neointimal fibrin deposits accounted for 17.8% of the neointima versus 0.9±1.4% of controls (\(P<0.02\), Figures 2A and 2B). Inflammatory cells were infrequent in both treatment groups; the percent of the neointima occupied by RAM11-positive macrophages was 0.021±0.051% in everolimus-treated rabbits compared with 0.044±0.051% in placebo-treated animals (\(P=NS\)). Neointimal cell density was reduced in everolimus stents (1470±941 cells/mm\(^2\) versus 2536±450 cells/mm\(^2\) in...
controls, $P<0.04$, Figure 3A). The neointimal cell proliferation labeling index was $1.87 \pm 1.01$ in everolimus stents compared with $0.82 \pm 0.49$ in controls ($P<0.03$, Figure 3B).

Twelve stents were deployed in 6 group 2 everolimus rabbits, and 12 stents were deployed in 6 placebo controls. Everolimus was associated with a significant reduction in neointimal thickness (42% reduction), neointimal area (24% reduction), and percent stent stenosis (25% reduction), with a 9% increase in lumen area (Table). The area within the internal elastic lamina was similar in everolimus and placebo animals. In contrast to group 1 everolimus rabbits, everolimus group 2 rabbits had similar medial area and thickness to placebo controls.

In most group 2 everolimus arterial segments, the intima was well healed, characterized by a compact intimal covering over stent struts and between struts (Figures 1F and 1G). Focal delayed healing was present around occasional struts, characterized by struts uncovered by neointima or focal neointimal fibrin deposition. The percent of the neointima occupied by fibrin in group 2 everolimus stents was less than group 1 everolimus stents ($P<0.03$) but remained greater than placebo ($4.5 \pm 4.9\%$ in group 2 everolimus versus $0.7 \pm 1.5\%$ in placebo $[P<0.04]$, Figure 2). Group 2 everolimus stents had a reduced mean intimal cell density ($2036 \pm 366$ cells/mm$^2$) compared with placebo stents ($2338 \pm 347$ cells/mm$^2$), but the difference did not reach statistical significance (Figure 3A). BrdU stains demonstrated a cell proliferation labeling index of $2.01 \pm 0.87\%$ in everolimus stents versus $0.89 \pm 0.28\%$ in placebo stents ($P<0.006$, Figure 3B). Inflammatory cells were rare; the percent of the

![Figure 1. Iliac artery morphology 28 days after stent placement. Low-power (A) and high-power (B, from box in A) views of a stent in a rabbit treated with placebo demonstrate a healed neointima (n) consisting of smooth muscle cells in a proteoglycan/collagen matrix. Inhibition of neointimal growth (C) was associated with variable healing in group 1 everolimus stents. Endothelial cells cover the neointima (D, from upper box in C), which is thinner but otherwise morphologically similar to the placebo stent. Incomplete healing associated with group 1 everolimus is shown in E (from lower box in C); the stent strut lacks neointimal coverage, and fewer smooth muscle cells are present around the strut. A group 2 everolimus stent (F and G) shows a healed thin neointima. (Movat pentachrome; scale bar=0.15 mm.]

![Figure 2. Bar graph (A) showing significantly increased neointimal fibrin deposition in animals treated with everolimus. The percent of the neointima occupied by fibrin was greatest in group 2 everolimus rabbits. Photomicrographs (B) demonstrate no neointimal fibrin in a placebo rabbit, a large fibrin deposit (red staining) in a group 1 everolimus animal, and a smaller deposit in a group 2 everolimus animal. (Antifibrin antibody staining with hematoxylin counterstain; scale bar=0.15 mm.)]
neointima occupied by RAM11-positive macrophages was 0.016±0.030% in everolimus rabbits compared with 0.038±0.037 in placebo animals (P=NS).

Scanning electron microscopy (Figure 4) demonstrated endothelial cell coverage of 86.7±6.8% of the stent surface in group 2 everolimus-treated animals versus 96.8±5.4% in placebo-treated rabbits (P<0.006). In everolimus-treated arteries, endothelial cell junctions were loose and easily separated during dehydration, especially close to the stent struts. Macrophages and platelets were focally adherent to nonendothelialized stent strut areas and over loosely formed endothelial junctions, particularly near stent struts in the midportion of the stent.

Arterial injury was mild in all treatment groups (mean injury score <1). Everolimus treatment (groups 1 and 2) was not associated with stent mal-apposition to the arterial wall or increased intimal growth in the nonstented proximal and distal arterial edge segments.

**Discussion**

Most systemic pharmacological therapies have been unsuccessful in preventing arterial restenosis in humans. Although some small trials have shown promising results, there are no convincing data that lipid-lowering drugs, antioxidants, anti-thrombotic agents, or angiotensin inhibitors are effective in reducing neointimal growth. Failure of systemic therapy to inhibit restenosis has been attributed to low antiproliferative potency of these agents and inadequate drug concentrations to inhibit the critical early steps in the restenosis process.
Disappointing results with systemic treatment and the ability to administer high local concentrations of potent antiproliferative therapy without systemic toxicity have driven the development of drug-eluting stents. Initial early clinical trials with sirolimus and paclitaxel stents are promising. However, the challenges entailed by stent-based drug delivery are numerous: the selection of a potentially effective drug, drug solubility, dosage, polymer coating, local pharmacokinetics (elution rates, arterial tissue concentration, and retention), and stent type (open- versus closed-ring designs). Additionally, local therapy may delay rather than prevent neointima. This is supported by preclinical studies that show impaired healing and neointimal catch-up. There is concern that neointimal growth will accelerate in response to the nonbiodegradable polymer coating after complete elution of the drug. These issues may revitalize investigations into systemic therapy, particularly with those agents that have shown positive results when administered locally, as stand-alone therapy, or as an adjunct to drug-eluting stents.

Everolimus, an orally active inhibitor of mTOR of the same macrolide family as sirolimus, inhibits growth factors-stimulated proliferation of hematopoietic and nonhematopoietic cells. In vitro, subnanomolar concentrations of everolimus inhibit the proliferation of bovine vascular smooth muscle cells to a similar degree as sirolimus. Although the immunosuppressive activity of everolimus is 2- to 5-fold lower than sirolimus in vitro, oral everolimus is at least as potent as sirolimus in models of autoimmune disease and heart and kidney allotransplantation. Oral everolimus is associated with reduced intimal thickening of aortic orthotopic transplants in rats.

In the present study, oral administration of everolimus demonstrated a significant reduction in in-stent neointimal growth at 28 days. In the higher-dose everolimus group (group 1), there was a 49% reduction in mean neointimal thickness and a 36% reduction in percent stent stenosis. Inhibition of neointimal formation was slightly less pronounced for the lower-dose everolimus animals (group 2), but there remained a significant 40% reduction in mean intimal thickness and a 26% reduction in percent stent stenosis. The results of group 2 are particularly promising, because this lower-dose regimen was both efficacious in neointimal growth suppression and well tolerated by the animals. The degree of in-stent neointimal inhibition was similar to that seen with the use of sirolimus-eluting stents (albeit in porcine coronary arteries with higher arterial injury scores). Thus, everolimus might potentially provide drug-delivery stent-like results without the engineering challenges and potential costs of manufacturing a drug-eluting stent. An alternative application of effective systemic therapy is the ability to provide follow-up booster dosing once local stent-based drugs are exhausted (ie, completely released from the stent).

Although the intimal surface was not fully healed in the group 2 everolimus rabbits and neointimal cell proliferation remained greater than in controls, most arteries showed near-complete healing, with a low local toxicity profile characterized by intimal coverage of most stent struts by a thin neointima (smooth muscle cells with surrounding matrix), overlying endothelial cells, few inflammatory cells, and no stent mal-apposition. The percent neointima occupied by fibrin was greater in everolimus stents versus placebo controls but accounted for <5% of the total neointimal area. In contrast, sirolimus-eluting stents in porcine coronary arteries also showed a significant increase in neointimal fibrin, which occupied up to 25% of the circumference of the artery. The state of healing was greater with oral everolimus as administered to group 2 animals than that observed at 28 days with paclitaxel-eluting stents in the same animal model, in which there was a significant increase in intra-intimal hemorrhage and increased inflammation.

With respect to stent endothelial coverage, >80% of the stent surface in everolimus-treated animals was endothelialized by 28 days (group 2). In contrast, with vascular brachytherapy, only 33% of the intimal surface of 6-μCi β-emitting stents is endothelialized at 3 months, and the degree of delayed healing with vascular radiation therapy is far greater (increased intimal fibrin deposition and inflammation) than that observed with everolimus in the present study. To our knowledge, no other studies of drug-eluting stents have assessed the extent of stent endothelial coverage at 28 days by scanning electron microscopy. Even with a semiquantitative light microscopic method, the stent endothelialization score of porcine coronary arteries with sirolimus-eluting stents was 2.9, corresponding to <75% stent surface endothelial coverage.

Limitations
It is unknown whether everolimus would prevent restenosis in an animal model with more severe vascular injury or in an atherosclerotic model. Longer-term studies are needed to determine if neointimal inhibition is prevented rather than delayed as neointimal cell proliferation remains elevated at 28 days.

Conclusion
Oral dosing with everolimus reduces in-stent neointimal growth in the rabbit iliac artery model. Oral administration of everolimus as an adjunct to sirolimus-eluting or other drug-eluting stents is an additional potential application of this agent and warrants further evaluation.

References


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