Dose-dependent Inhibition of Myointimal Hyperplasia by Orally Administered Rapamycin

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Myointimal hyperplasia (MIH) after vascular intervention is a major problem. Recent reports describing elimination of within-stent restenosis by means of rapamycin-eluting stents prompted us to examine the effect of systemic oral rapamycin on MIH induced by arterial trauma. We studied the effect of oral rapamycin on MIH after rabbit aorta balloon injury. Thirty-five New Zealand white rabbits (2.5-3 kg) had aortic injury and were given either no rapamycin (control), 0.1 (low dose) rapamycin mg/kg/day, or 0.4 mg/kg/day (high dose). Rapamycin was started 1 week before injury and continued for 3 (4 weeks total) or 6 weeks (7 weeks total) post-injury. Sections were analyzed to measure aortic intima/media area ratios (I:M) at either 3 or 6 weeks. At 3 weeks, the I:M (mean ± SD) for controls was 0.53 ± 0.1; for low dose, 0.17 ± 0.13; and for high dose, 0.24 ± 0.07 ($p < 0.001$ vs. control). At 6 weeks, the I:M for controls was 0.52 ± 0.12; for low dose-4 weeks, 0.29 ± 0.15; low dose-7 weeks, 0.33 ± 0.07; and high dose-4 weeks, 0.47 ± 0.16. At 6 weeks only the difference between the low dose-4 weeks and control I:M ratios was significant ($p = 0.018$). The results confirm earlier studies showing that systemic rapamycin inhibits MIH after arterial injury when drug therapy is started before injury. Therapy for 3 or 6 weeks after injury yields similar inhibition, indicating that exposure to the drug early in the response to injury is more important than prolonged exposure. We observed a paradoxical relation between dose and degree of MIH inhibition, with the low dose being more effective than the high dose at both time intervals studied. Overall, the results suggest that oral rapamycin therapy might be a useful adjunct to clinical interventions at risk for development of MIH.

INTRODUCTION

Myointimal hyperplasia (MIH) after vascular interventions is a major cause of delayed therapeutic failure. Late restenosis, for example, occurs in 20 to 30% of patients undergoing coronary balloon angioplasty and stenting.[1] In our experience, stents deployed to correct femoropopliteal occlusion developed severe MIH at 6 months in approximately half the patients, and the MIH worsened progressively and was the primary cause of failure. (Ian Gordon, unpublished observations). The development of MIH is a complex phenomenon resulting from arterial wall trauma. Platelet-derived growth factor (PDGF) and thrombus have been prominently implicated in this stimulation, but other factors appear likely to be involved.[2,3] Injury stimulates medial smooth muscle cells to undergo proliferation, dedifferentiate with expression of fetal phenotype proteins, and migrate into the intima. The morphologic changes in ultrastructure entail both hyperplasia of the migrant smooth muscle cells and enhanced deposition of extracellular matrix proteins and glycosoaminoglycans,[4-6] leading to thickening of the intimal layer and narrowing of the arterial lumen.

Methods to limit MIH include the use of external and local intraluminal ionizing radiation[7] and the

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use of drug-eluting stents.[8] The latter approach avoids certain safety and technical problems with radiation or isotope therapy. Recent clinical studies indicate that nearly complete elimination of within-stent restenosis may be achieved in coronary stents impregnated with rapamycin, a macrocyclic antibiotic derived from *Streptomycetes hygroscopius*.[9,10] Rapamycin's inhibitory effect is mediated by initial binding to cytosolic proteins, most prominently the FK506 binding protein FKBP12, which is expressed at high levels in many eukaryotic cells with marked sequence homology.[11,12] The rapamycin-FKBP complex associates with and inhibits the activity of a protein with phophatidyl-inositol kinase activity.[13,14] known as the mammalian target of rapamycin (mTOR). mTOR inhibition prevents phosphorylation of proteins involved in regulation of the cell cycle leading to arrest at the G1/S interface[15,16] and inhibition of intracellular signaling pathways associated with growth factor stimulation.[17,18] In addition to the effects observed on fibroblasts, immune cells, and hepatocytes, rapamycin inhibits vascular smooth muscle cell proliferation and migration as well as intimal thickening after injury.[19,20]

Oral bioavailability of rapamycin is 5% in rabbits and is somewhat higher in humans, reaching 15% with concurrent administration of cyclosporine.[21] It is sensitive to degradation by P450 3A (CYP3A) enzymes in both intestinal mucosa and liver, with the common metabolites being hydroxylated, demethylated, or both.[22] A significant proportion of orally administered rapamycin is degraded by cytochrome metabolism in the intestinal mucosa.[23] Native rapamycin is much more effective in inhibiting cellular immunity than its common metabolites. Elimination is primarily fecal and biliary, with minimal renal excretion.[24] In addition to suppression of transplant organ rejection, rapamycin causes increased susceptibility to infection as well as hyperlipidemia.[25] High doses of rapamycin in rabbits (1 mg/kg/day) lead to weight loss, decreased food intake, decreased intestinal mucosa villus surface area, and decreased intestinal absorption of nutrients.[26]

We hypothesized that systemic administration of rapamycin would inhibit intimal hyperplasia after balloon injury of the aorta in normocholesterolemic rabbits, a model familiar to us from previous studies.[27] We were particularly interested in the influence of dose and duration of administration on inhibition of MIH. If effective, oral rapamycin therapy would be of potential value when impregnated stent deployment was not feasible—for example, to reduce intimal hyperplasia in infrainguinal saphenous vein grafts.[28]

MATERIALS AND METHODS

Pediatric rapamycin solution (sirolimus, Rappamune®) with 1 mg rapamycin/mL and containing a mixture of lipids, soy fatty acids, and ethanol in an aqueous vehicle was purchased from Wyeth (Madison, NJ). New Zealand white rabbits (2.5-3 kg) were purchased from Western Oregon Rabbit Co. (Philomath, OR). Animals were housed in accordance with National Institutes of Health (NIH) guidelines and experimental procedures involving animals were approved by the Animal Care Committee of University of California, Irvine. Rabbits avidly ingested the rapamycin solution, which was gently introduced with a 1-mL syringe into their mouths. Intramuscular 5 mg/kg xylazine (5 mg/mL) and ketamine (50 mg/kg) were used for surgical anesthesia. Aortic balloon injury was induced with a 2-Fr Fogarty balloon embolectomy catheter (Baxter, Irvine, CA) passed 15 cm proximally through an arteriotomy in a surgically exposed femoral artery. The balloon was inflated with saline to generate resistance, withdrawn distally 10 cm, and deflated, and the abrasion was repeated twice.

Groups of five rabbits receiving either dose (0.1 or 0.4 mg/kg daily) of rapamycin had drug administration invariably started 1 week before balloon injury. For characterization of aortic MIH at 3 weeks, rapamycin administration was maintained until 24 hr before sacrifice. There were four groups of rabbits in which aortic morphology was characterized 6 weeks after injury: a control group receiving no drug; a low-dose group receiving drug for 3 weeks after injury (low dose-4 weeks); a low-dose group receiving drug for 6 weeks after injury until sacrifice (low dose-7 weeks), and a high-dose group receiving drug for 3 weeks after injury. Body weights were measured twice weekly.

Abdominal aortas were perfused in situ with 10% phosphate-buffered (pH 7.4) formaldehyde solution under physiological pressure. A 2-cm segment of the aorta just proximal to the iliac bifurcation was excised, fixed overnight in formalin, and then embedded in paraffin. Six-micron-thick microtome sections were stained with either hematoxylin and eosin or elastin stain for histological analysis. Photomicrographs of elastin-stained sections were acquired with a digital camera (DP-10; Olympus, Tokyo, Japan) mounted on a microscope (4× objective), saved as TIFF files, and subsequently analyzed for cross-sectional intima and media areas with NIH image software. Measurements from three elastin sections, taken from three positions in each aorta separated by 5-6 mm, were averaged to calculate the intima/media

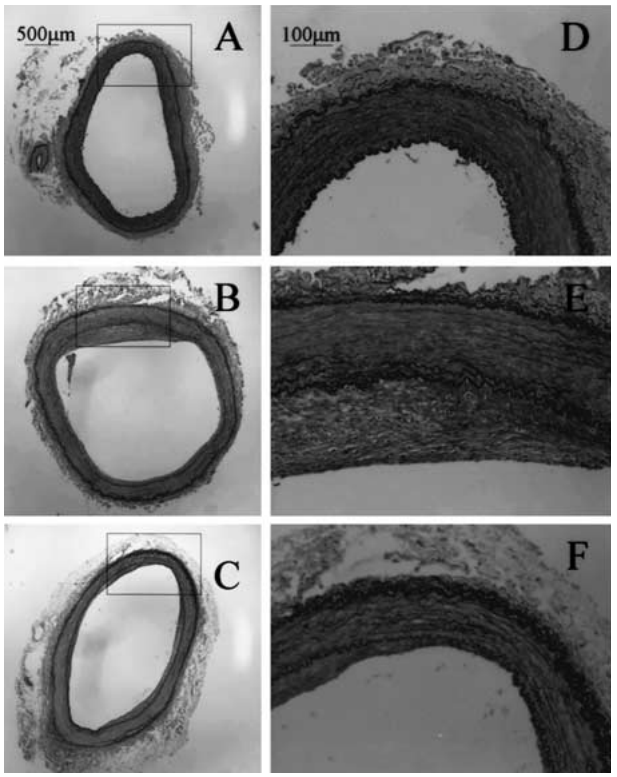

Fig. 1. Representative photomicrographs of elastin-stained, 6-μm sections from normal rabbit aorta (**A, D**), control rabbits 3 weeks after balloon injury (**B, E**), and rapamycin-treated rabbit aorta 3 weeks after injury (**C, F**).

(I:M) area ratio for each rabbit. Data are expressed as mean ± standard deviation (SD). Comparison of morphologic measurements such as intima area or I:M ratios was performed by analysis of variance (ANOVA) with Fisher's *t* test.

RESULTS

Representative photomicrographs of a normal aorta and of MIH developing 3 weeks after balloon injury in control and rapamycin-treated animals are shown in Figure 1. The intima layer in normal rabbits is too small to be accurately measured by light microscopy, and the characteristic response to balloon injury was a dramatic thickening that led to an intimal layer many times thicker than that seen in uninjured vessels.

As enumerated in Table I, at 3 weeks, both doses of rapamycin markedly reduced measured intima area ($p < 0.01$) compared to controls not receiving drug. At 6 weeks the effect of rapamycin therapy on intima thickening was less dramatic, with no inhibition evident in the high-dose rapamycin group, and the differences between the control and both low-dose groups (4 or 7 weeks total rapamycin therapy) did not achieve statistical significance.

The changes in media thickness observed after balloon injury and with rapamycin therapy were somewhat more complex. At 3 weeks, compared to normal rabbits, balloon injury stimulated an approximate 41% increased thickness of the medial layer. Rapamycin therapy limited the increase in medial thickness seen at 3 weeks; low-dose rapamycin therapy led to an approximate 4% increase in media area and high-dose therapy led to a 10% increase in media area. At 3 weeks, the difference in media area between control rabbits and either normal or rapamycin-treated rabbits was significant ($p < 0.01$), but not the difference between normal and rapamycin-treated rabbits. At 6 weeks, the media area of control rabbits was not increased as much as at 3 weeks, and the difference in area between controls and normals was not significant. In contrast to the finding at 3 weeks, however, the media area was greater in all rapamycin-treated groups than in controls; the differences between the high-dose group and controls and between low dose-7 weeks rapamycin-treated rabbits and controls were significant ($p < 0.01$).

When I:M ratios were determined (Fig. 2), at 3 weeks there was an average of 68% inhibition of the I:M ratio in the low-dose group and 55% in the high-dose group, compared to controls ($p < 0.001$). At 6 weeks, significant inhibition of the I:M ratio was induced only in the low-dose regimens, with a 44% reduction in the ratio associated with the low dose-4 weeks group ($p < 0.01$) and a 36% reduction in the ratio in the low dose-7 weeks group ($p < 0.05$). With the higher rapamycin dose, only a nonsignificant 8% inhibition of the I:M ratio was evident at 6 weeks after injury. Endothelial healing of the injured aortic segments appeared complete in all treatment groups by 3 weeks, and residual thrombus or fibrin deposits were not noted in any specimen.

There was no difference in weight gain observed between the various groups, nor did we see evidence of systemic toxicity, effects associated with doses of 1 mg/kg in rabbits. Subsequent studies with the 0.1 mg/kg dose (data not shown) have resulted in no bone marrow suppression.

DISCUSSION

In our rabbit model for MIH, oral rapamycin started before injury at two doses (0.1 mg/kg/day and 0.4 mg/kg/day) and continued until sacrifice at 21 days induced marked inhibition of intimal hyperplasia, as manifested by the I:M ratio. An unex-

Table I. Planimetry measurements

Group	*n*	Intima (mm^2) (mean ± SD)	Media (mm^2) (mean ± SD)	I:M ratio (mean ± SD)
Normal	4	—	0.78 ± 0.09	—
3 Weeks				
Control	5	0.58 ± 0.11	1.1 ± 0.17	0.53 ± 0.1
Low dose	5	0.15 ± 0.14[a]	0.81 ± 0.14[a]	0.17 ± 0.13[a]
High dose	5	0.2 ± 0.52[a]	0.86 ± 0.72[a]	0.24 ± 0.07[a]
6 Weeks				
Control	5	0.46 ± 0.19	0.87 ± 0.18	0.52 ± 0.12
Low dose (rapamycin for 4 weeks)	5	0.29 ± 0.15[b]	0.99 ± 0.11[b]	0.29 ± 0.15[a]
High dose (rapamycin for 4 weeks)	5	0.55 ± 0.18[c]	1.16 ± 0.17[a]	0.48 ± 0.16[d]
Low dose (rapamycin for 7 weeks)	5	0.37 ± 0.12[e]	1.11 ± 0.17[a]	0.33 ± 0.07[f]

[a] $p < 0.01$ vs. control.
[b] $p = 0.15$ vs. control.
[c] $p = 0.47$ vs. control.
[d] $p = 0.55$ vs. control.
[e] $p = 0.44$ vs. control.
[f] $p < 0.05$ vs. control.

pected and paradoxical dose effect was discovered in that the low dose was more effective than the high dose (68% vs. 55% reduction in I:M ratio) at 21 days. When assessment of MIH was performed 42 days after injury, however, only the low dose exerted significant (44%) inhibition when administered through day 21, compared to a modest and insignificant 8% reduction in the I:M ratio associated with high dose delivered for the same interval. Prolonged administration of rapamycin at the low dose through sacrifice at day 42 (7 weeks total therapy) yielded slightly less inhibition (36%) than that achieved with the shorter course of low-dose therapy. This indicates that rapamycin inhibition of MIH depends on effects exerted immediately after mechanical injury and that prolonged exposures are not increasingly effective. The observation that less inhibition of MIH was present at 42 days than at 21 days is consistent with other experiments with arterial injury models. Often drugs that markedly inhibit hyperplasia early appear less effective later, as remodeling of the injured artery shows a ''catch-up'' phenomenon, and the difference between treatment and control groups becomes less pronounced.[29] Overall, our results are consistent with previous demonstrations that systemic rapamycin has the ability to significantly inhibit intimal hyperplasia after balloon injury, stenting, or allogeneic transplantion in animal models.[20,30,31]

The ability of relatively brief systemic rapamycin administration to inhibit MIH is consistent with clinical observations that rapamycin-eluting stents achieve comparable results with either 14 or 28 days of drug release.[9] The apparent critical need for early exposure of the injured artery to rapamycin for inhibition of MIH to occur is supported by previous observations that even single doses at the time of transplant can inhibit intimal hyperplasia in an allograft injury model.[32] Systemic rapamycin inhibition of smooth muscle cell migration stimulated by PDGF is much more effective when therapy is started 2 days prior to PDGF exposure, rather than the day of injury.[19] Similarly, when systemic rapamycin was started 3 days before balloon injury in a rat model, inhibition of MIH was much more effective than that achieved with drug administration started on the day of injury.[33]

Previous studies of rapamycin- or everolimus-impregnated stents in animal models have shown that, compared to controls, local drug delivery inhibited within-stent stenosis or intimal hyperplasia by approximately 50%, but not much more.[34-36] Clinical trials with rapamycin-impregnated coronary stents have found within-stent restenosis to be nearly completely eliminated, indicating that atherosclerotic lesions may be more sensitive to rapamycin than normal animal arteries.[37] Human leukocytes are more sensitive to the antiproliferative effect of rapamycin than porcine leukocytes,[38] and the clearance of systemic rapamycin is slower in humans than in other species.[39] Overall, both the current study and previous reports of systemic therapy with rapamycin in animals support the potential feasibility of employing short courses of oral rapamycin, possibly in combination with other drugs, to limit MIH in clinical situations where impregnated stents are not a practical intervention.

Our study shows a paradoxical effect of rapamycin dose on inhibition of MIH, with the high

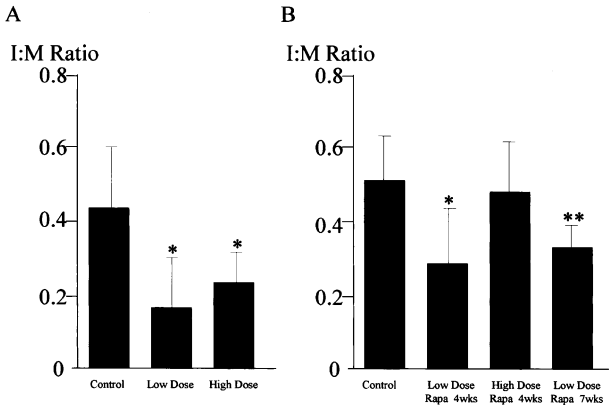

Fig. 2. Effect of rapamycin on I:M ratios at 3 weeks (**A**) and 6 weeks (**B**). The bar graph shows the decrease in I:M ratio induced by drug therapy for each treatment group. $*p < 0.01$, $**p < 0.05$ vs. control.

dose being less effective. This type of inverse relation has not been demonstrated in previous studies of either in vivo or in vitro effects of rapamycin on intimal hyperplasia, smooth muscle cell proliferation, or migration. Although the regimen for systemic rapamycin used here has not been previously described, we cannot easily explain this unexpected effect. We doubt that the high-dose (0.4 mg/kg/day) observations reflect metabolic effects due to malnutrition, as no reduction in weight gain was observed, as has been previously described with 1 mg/kg/day dosing in rabbits.[26] We surmise that at the higher dose, other effects of rapamycin on the development of intimal hyperplasia come into play, perhaps a species-specific ''inhibition of inhibition'' or tachyphylaxis. In future studies, we intend to examine the effect of lower rapamycin doses—conceivably they will be as effective as the 0.1 mg/kg dose. Other topics of interest are elucidation of the optimum duration for rapamycin therapy, and the interactions between rapamycin and other drugs that inhibit MIH.

CONCLUSIONS

Oral rapamycin administration causes potentially useful inhibition of MIH. Paradoxically, low doses may be more effective than high doses, and inhibition is not necessarily enhanced by longer periods of oral administration.

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