Rapamycin Limits the Growth of Established Experimental Abdominal Aortic Aneurysms

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WHAT THIS PAPER ADDS
Abdominal aortic aneurysms generally enlarge and rupture unless resected or repaired. To date, pharmacological strategy has proven ineffective in preventing disease progression. In this study, rapamycin proved remarkably effective in preventing progression of established experimental aneurysms. Despite beginning therapy after aneurysm initiation, rapamycin preserved aortic architecture, and attenuated aortic mural angiogenesis and macrophage accumulation. This study adds to the growing body of evidence supporting the use of rapamycin for medical abdominal aortic aneurysm disease management.

Objectives: Abdominal aortic aneurysm (AAA) is a chronic inflammatory disease affecting 4—8% of men older than 60 years. No pharmacologic strategies limit disease progression, aneurysm rupture, or aneurysm-related death. We examined the ability of rapamycin to limit the progression of established experimental AAAs.

Methods: AAAs were created in 10—12-week-old male C57BL/6J mice via the porcine pancreatic elastase (PPE) infusion method. Beginning 4 days after PPE infusion, mice were treated with rapamycin (5 mg/kg/day) or an equal volume of vehicle for 10 days. AAA progression was monitored by serial ultrasound examination. Aortae were harvested for histological analyses at sacrifice.

Results: Three days after PPE infusion, prior to vehicle or rapamycin treatment, aneurysms were enlarging at an equal rate between groups. In the rapamycin group, treatment reduced aortic enlargement by 38%, and 53% at 3 and 10 days, respectively. On histological analysis, medial elastin and smooth muscle cell populations were relatively preserved in the rapamycin group. Rapamycin treatment also reduced mural macrophage density and neoangiogenesis.

Conclusion: Rapamycin limits the progression of established experimental aneurysms, increasing the translational potential of mechanistic target of rapamycin-related AAA inhibition strategies.

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INTRODUCTION
Abdominal aortic aneurysm (AAA) is a lethal, age- and gender-related chronic inflammatory disease. Approximately one million Americans aged 50—84 years are at risk for premature, AAA-related death. Key pathological features present in AAA disease include mural leukocyte infiltration, neoangiogenesis, smooth muscle cell (SMC) depletion, extracellular matrix degradation, and progressive intraluminal laminar thrombus accumulation. Although multiple pharmacological inhibition strategies have succeeded in experimental aneurysm models, none have been successfully translated to clinical practice. Therefore, surgical repair, limited to patients suffering from advanced disease, remains the only available option.

Rapamycin is approved by the US Food and Drug Administration for preventing rejection of transplant allografts, as well as coronary artery restenosis following transluminal angioplasty and stenting. Mechanistically, rapamycin disrupts cellular signaling through mechanistic/mammalian target of rapamycin (mTOR), a serine/threonine kinase modulating numerous biological processes, including cell growth, metabolism, aging, angiogenesis, and inflammation. In experimental atherosclerosis, rapamycin treatment increases plaque stability and limits disease progression. Rapamycin is effective in limiting experimental aneurysm progression when administered prior to AAA initiation. However, of critical relevance to translational potential, the ability of rapamycin to limit existing aneurysm progression has not been examined to date.

In this study, rapamycin therapy was initiated 4 days after intra-aortic infusion of porcine pancreatic elastase (PPE), at
which point the aneurysmal degeneration has been initiated. Rapamycin is remarkably effective in limiting progression in this construct, further highlighting the translational potential of this therapeutic strategy.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice aged 10–12 weeks were used for all experiments. All animal protocols were in compliance with the Laboratory Animal Care Guidelines of Stanford University, and reviewed and approved by the Stanford University Administrative Panel on Laboratory Animal Care.

Aneurysm creation and follow-up

AAAs were created via intra-aortic PPE infusion as previously described.\(^8,22\) Briefly, under inhaled anesthesia and under operative magnification, 30 μL of type 1 PPE (1.5 U/mL in saline, Catalog # 098K7008; Sigma-Aldrich, St. Louis, MO, USA) was infused for 5 minutes into an isolated segment of infrarenal aorta. At days 3, 7, and 14 after the infusion procedure, the aortic diameter was measured serially using the Vevo 770 ultrasound system (40 MHz; VisualSonics, Toronto, ON, Canada).\(^8,22\) Two investigators performed all measurements independently, without knowledge of study group assignment.

Rapamycin treatment

Rapamycin was purchased from the LC Laboratories (Woburn, MA, USA) and prepared in 0.2% carboxy-methylcellulose immediately prior to use. Mice were treated daily with 5 mg/kg rapamycin or an equal volume of vehicle alone via oral gavage, depending on study group assignment, beginning 4 days after PPE infusion and continuing for 10 days. This dose was chosen based on a literature review of published work in atherosclerosis models.\(^17–19\)

Histological analyses

Aortae were harvested 14 days after PPE infusion, embedded in optimal cutting compound media, sectioned (6 μm), and fixed with cold acetone. Elastin integrity was evaluated using Verhoeff’s Van Gieson (EVG) stain. SMCs, macrophages, and angiogenesis were stained with antibodies against SMC α actin, CD68, and CD31, respectively, using a standard three-step biotin–streptavidin–peroxidase immunostain.\(^8,22\) Based on EVG and SMC α actin staining, destruction of medial elastin and SMCs were graded as I (mild) to IV (severe) if present:8,22 (I): elastin break/degradation or SMC loss limited to one outer medial elastin layer; (II): elastin degradation or SMC loss involving more than two medial elastin layers, or entire medial elastin layers, but limited to less than one-quarter of the aortic circumference; (III): elastin degradation or SMC loss involving entire medial elastin layers, but limited to less than half the aortic circumference; and (IV): elastin degradation or SMC loss involving entire medial elastin layers and expanded to more than three-quarters of aortic circumference. Mural macrophages and angiogenesis were quantified as CD68\(^+\) cells and CD31\(^+\) blood vessels, respectively, per aortic cross section (ACS).

Statistical analysis

All data are presented as mean and standard deviation (SD), and were analyzed using GraphPad Prism (version 5a; GraphPad Software, La Jolla, CA, USA). Two-way ANOVA (using the Bonferroni correction for multiple comparisons) or the non-parametric Mann–Whitney test was used to identify differences between groups. Significance was assumed at \(p < .05\).

RESULTS

Effects of rapamycin treatment on further progression of established aneurysms

In our prior experience with this model, most mice develop characteristic histological features and > 50.0% infrarenal diameter enlargement consistent with AAA formation within 3–4 days of PPE infusion.\(^8\) To evaluate the ability of rapamycin to limit progression of established AAAs, mice were treated with 5 mg/kg/d rapamycin via oral gavage, beginning 4 days after the PPE infusion and continuing for 10 days in total. Prior to drug or vehicle treatment (3 days after PPE infusion), five and six mice in the vehicle and rapamycin groups, respectively, developed AAAs as defined by a > 50.0% increase in aortic diameters over the baseline levels. As demonstrated in Fig. 1, rapamycin treatment was associated with a small, but significant, loss of weight during the course of the experiment (103.8 ± 4.9% and 97.2 ± 3.6% of baseline levels in vehicle and rapamycin groups, respectively; \(p < .05\)). Fig. 2 demonstrates the time-dependent progression of aortic diameter, as determined by ultrasound imaging. In vehicle-treated mice, aortic diameters increased by an average of 0.13 and 0.36 mm between days 3 and 10, respectively. In contrast, treatment with rapamycin resulted in remarkable attenuation of

![Figure 1. Effect of rapamycin treatment on body weight. Data on body weight are presented as the percentage of baseline levels (prior to vehicle or rapamycin treatment). Two-way analysis of variance with the Bonferroni correction, \(p < .01\) between two groups; \(n = 7–8\) mice/group.](image-url)
further enlargement, with growth averaging 0.07 and 0.17 mm for days 3 and 10, respectively, corresponding to a 38.0–53.0% reduction (p < .05 compared with vehicle group). In this context, therefore, rapamycin treatment appears effective in limiting further progression of established experimental AAA.

Effects of rapamycin treatment on medial elastin and SMCs

Medial elastin destruction and SMC depletion are hallmarks of AAA histopathology. To examine the influence of rapamycin therapy on aortic structural integrity, EVG and SMα-actin staining was performed on aortic samples (Fig. 3). As indicated by semi-quantitative analysis, rapamycin treatment resulted in significant aortic SMC retention compared with vehicle treatment alone (p < .05). Further confirmation was obtained by counting SMα-actin-positive cells in the aortic media (56.3 ± 8.4 and 32.7 ± 16.2 cells/ACS in rapamycin and vehicle-treated groups, respectively; p < 0.05). Although some aortic elastin preservation was apparent in rapamycin-treated mice, this difference was not significant. These findings suggest a potential mechanism for rapamycin-induced AAA suppression, specifically augmentation of aortic SMC retention/preservation.

Effects of rapamycin treatment on mural macrophage infiltration and angiogenesis

Macrophage accumulation and mural neoangiogenesis also represent characteristic histopathologic features of abdominal aortic degeneration. Mural macrophage density and angiogenesis was examined in frozen aortic sections using CD68 and CD31 monoclonal antibody immunostaining, respectively. Numerous mural macrophages were present in the aneurysmal aortae of vehicle-treated mice (352 ± 125 cells/ACS) (Fig. 4A, B). In rapamycin-treated mice, macrophage density was reduced by 75.0% (90 ± 57 cells/ACS, p < .01). Similarly, adventitial CD31+ neovessel density was significantly lower (3 ± 3 blood vessels/ACS) than in vehicle-treated mice (19 ± 12 blood vessels/ACS), representing an 85.0% reduction (Fig. 4A, C) (p < .01). These results suggest, in addition to effects on SMC preservation, that rapamycin therapy may limit aneurysmal degeneration via suppression of mural macrophage accumulation and adventitial neoangiogenesis.

DISCUSSION

In this study, we found rapamycin to be remarkably effective in preventing the progression of established aneurysms. Rapamycin-induced aneurysm inhibition was accompanied by relative preservation in aortic architecture and reduced inflammation. These results lend further support to existing data supporting rapamycin as a prime translational candidate in early AAA disease suppression.20,21

Macrophages are likely the principal effector cells of aneurysmal disease, contributing to aortic degradation via the production of extracellular matrix-degrading proteases, lipid mediators, reactive oxygen species, and proinflammatory cytokines.23–25 Aortic hemodynamic conditions modulated aortic mural macrophage density and aneurysm progression,26 as well as deficiency or inhibition of the chemokine receptor CCR2 or its ligand CCL2.27–31 More recently, the cyclic peptide MKEY, an inhibitor of CCL5–CXCL4 chemokine interaction, was also found to limit experimental aneurysm progression while reducing mural monocyte migration and macrophage density.8

The effect of rapamycin treatment on mural inflammation and aneurysm initiation/progression has been previously investigated to varying degrees. Prior experiments have demonstrated the ability of rapamycin pretreatment to subsequently suppress aneurysm progression following subsequent AAA initiation, without apparently influencing inflammatory cell infiltration.20 Discrepancy between those findings and the results of the current study may be owing to the fivefold higher rapamycin dose employed in the latter (although also administered by oral gavage).

Mural macrophage density is the summative end-organ consequence of several related cellular processes, including monocyte mobilization from systemic stores in the bone marrow and spleen, transendothelial migration from the bloodstream to inflamed aorta, and in situ macrophage proliferation, retention, egress, and cell death.32,33 In patients with chronic stable angina, the use of rapamycin-coated...
coronary stents was associated with reduced serum CCL2 levels. Rapamycin down-regulated monocyte CCR2 and CCR5 expression, and inhibited in vitro monocyte migration towards CCL2 and other chemoattractants. Rapamycin also inhibited vascular endothelial growth factor (VEGF)-A production, another factor regulating influence on monocyte migration. In congenitally hyperlipidemic mice, pretreatment with rapamycin was recently shown to reduce aneurysm formation after exogenous angiotensin (Ang) II infusion, in conjunction with a reduction in circulating monocytes, and monocyte CCR2 expression. Thus, despite earlier findings to the contrary, rapamycin may limit experimental aneurysm initiation/progression through influences on monocyte mobilization, migration, or tissue differentiation.

In this study, rapamycin also reduced aortic mural neovascularization in experimental AAAs. Although we observed a trend that reduced aortic neovascularization correlated with the attenuation of aortic enlargement to a certain extent, it did not reach statistical significance (Spearman’s rank correlation = 0.6, p > .05, data not shown). While the significance of this finding may have been obscured by insufficient power, given the number of mice examined in this experiment, abundant prior observations link mural angiogenesis and AAA progression. Angiogenesis is tightly regulated by the transcription factor, hypoxia inducible factor (HIF-1α). In addition to hypoxia, growth factors, inflammatory cytokines, and reactive oxygen species also modulate aortic cellular expression of HIF-1α (and VEGF-A) in AAA disease. Because cellular levels vary, in part, owing to mTOR-mediated signaling pathways, rapamycin may attenuate AAA progression through influences on HIF-1α-mediated aortic mural angiogenesis.

Several alternative immunosuppression reagents have been shown to prevent experimental AAA formation, and Figure 3. Effects of rapamycin treatment on elastin and smooth muscle cell degradation. Aortic frozen sections prepared from mice 14 days after porcine pancreatic elastase infusion were stained with Verhoeff’s Van Gieson or anti-smooth muscle cell (SMC) α actin antibody and graded as I (mild) to IV (severe). (A) Representative staining images for medial elastin and SMCs. Scale bar = 100 μm. (B, C) Mean and standard deviation of the scores for SMC (B) and medial elastin (C) destruction. Mann—Whitney test, *p < .05 compared with vehicle group; n = 7–8 mice/group.
azathioprine and cyclosporine specifically prevent the progression of established AAAs. Interestingly, however, in patients receiving these drugs for long-term immunosuppression, existing AAA growth has been unaffected or even accelerated. Rapamycin may fare differently in this regard. For example, in addition to its influences on aortic mural macrophage density and neoangiogenesis, rapamycin inhibits T cell production of interferon-γ and interleukin-17, important mediators of AAA progression, and induces regulatory T cells, a negative regulator for aneurysm disease. Rapamycin also diminishes classically activated macrophage polarization in experimental aneurysm models.

Finally, we and others have demonstrated the critical role of Ang II receptor in AAA pathogenesis, even in models created without exogenous Ang II administration. Among many other pro-aneurysmal influences on the aorta, Ang II also activates the mTOR pathway in vascular constitutive cells. Thus many properties of rapamycin, apart from its known influences on immunological competence, may account for its effectiveness in experimental (and potentially clinical) AAA suppression.

Because the primary goal of these experiments was to examine the influence of rapamycin on the progression of established AAAs, some limitations exist regarding the generalizability of these results. As has been reported previously in the experimental literature, the dose of rapamycin employed (5 mg/kg/day) was far higher than the standard human clinical dose (up to 40 mg daily [<1 mg/kg/day], and more typically 2–5 mg daily for maintenance dosing) (USPI guide for oral rapamune/sirolimus therapy; Pfizer, Philadelphia, PA, USA). The question of whether rapamycin, in the clinical dosing range, is effective at limiting experimental AAA progression was not specifically

Figure 4. Effects of rapamycin treatment on mural angiogenesis and macrophage accumulation. Aortic frozen sections prepared from mice 14 days after porcine pancreatic elastase infusion were stained with monoclonal antibodies against CD31 and CD68. (A) Representative immunostaining images for CD68⁺ macrophages and CD31⁺ blood vessels. Scale bar = 100 μm. (B, C) Mean and standard deviation of mural macrophages (B) and angiogenesis (C) quantified as CD68⁺ macrophages or CD31⁺ blood vessels per aortic cross section (ACS). Mann–Whitney test, **p < .01 compared with vehicle group; n = 7–8 mice/group.
addressed in this study. Similarly, a dose-ranging experiment was also outside the scope of this investigation. Given its long half-time (approximately 60 hours), a reduced frequency of administration may also maintain efficacy at lower total doses. Thus, substantial work will be required before clinical trials can be justified in patients with early AAA disease.

Evaluation of the therapeutic efficacy of rapamycin was limited to transabdominal ultrasonography in vivo and aortic histological analyses. Further exploration of the cellular and molecular mechanisms responsible for the suppression of AAA progression by rapamycin are beyond the scope of this evaluation. Effects on aortic mural SMC preservation may be attributed to direct or indirect influences on apoptosis, survival, proliferation, and/or other regenerating properties of SMCs. Additionally, 10-day rapamycin treatment led to < 10.0% reduction body weight gain compared with vehicle treatment. Given the importance of body weight/mass on aneurysm growth, we cannot exclude the possibility that small reduced body weight gain by rapamycin may, in part, influence AAA growth.

CONCLUSION

Rapamycin limits progression of established experimental aneurysms, further supporting its translation potential in human AAA disease management.

CONFLICT OF INTEREST

None.

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