Sirolimus (Rapamycin) Monotherapy Prevents Graft Vascular Disease in Nonhuman Primate Recipients of Orthotopic Aortic Allografts

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Background—Delayed treatment with sirolimus (SRL) halts progression of graft vascular disease (GVD) in nonhuman primate (NHP) aortic allograft recipients. In this study, we investigated whether SRL monotherapy prevents the development of GVD.

Methods and Results—Pairs of 3-cm infrarenal aortic segments were exchanged between mixed lymphocyte reaction–mismatched, blood group–compatible NHPs (n=12). Six NHPs were untreated controls, and 6 were treated orally with SRL starting on the day of transplantation. Follow-up was 105 days. SRL doses were adjusted individually by assessing SRL blood concentrations, immune function, and clinical status. The severity of GVD was determined every 3 weeks by intravascular ultrasound, which quantified intimal area (IA) and intimal volume (IV) for the middle 1-cm graft segments. The mean±SEM SRL plasma levels were 14.5±9 ng/mL. In grafts from treated NHPs, IA and IV values on days 63, 84, and 105 were significantly lower than for controls (P<0.05 to P<0.001). On day 105, in the grafts from SRL-treated NHPs compared with grafts from controls, values (mean±SEM) were IA, 2.9±0.9 versus 5.5±0.7 mm², P<0.001 and IV, 29.6±4.6 versus 55.2±2.8 mm³, P<0.001; IA and IV values for grafts from SRL-treated NHPs did not increase significantly between days 21 and 105.

Conclusions—We show that SRL monotherapy prevented GVD in NHP aortic allograft recipients, suggesting the value of SRL for controlling GVD in clinical transplantation. (Circulation. 2003;107:2369-2374.)

Key Words: transplantation • arteriosclerosis • prevention • immunology

With the use of current immunosuppressive protocols, graft vascular disease (GVD) (manifested as progressive narrowing of the coronary arteries in the transplanted heart) remains the major cause of allograft loss long term after heart transplantation.1 Sirolimus (SRL) not only is a potent and effective immunosuppressant of allograft rejection2,3 but also inhibits intimal thickening after immune and mechanical injury by directly reducing the proliferation and migration of intimal smooth muscle cells (SMCs) in vitro and in vivo.4–10 We recently developed a new nonhuman primate (NHP) aortic allografting procedure in which the histopathological lesions of GVD are indistinguishable from those observed in human GVD.11 We previously demonstrated that delayed treatment with SRL halts progression of preexisting GVD in rats12 and NHP allografts.13

Our present study investigates whether de novo treatment with SRL alone prevents GVD in NHP recipients of aortic allografts.

Methods

Animals

Our study was approved by the Institutional Animal Care and Use Committee at Stanford University. Donor and recipient NHPs (male cynomolgus monkeys [Macaca fascicularis] weighing 6.3 to 8.3 kg, Charles River, Inc, Houston, Tex) were paired on the basis of ABO blood group match, negative red cell cross-match, and mixed lymphocyte reaction mismatch.

Phase I Study

A pharmacokinetic/pharmacodynamic tolerability study was conducted in 4 nontransplanted NHPs treated orally with 1.5 mg · kg⁻¹ · d⁻¹ of SRL over a period of 7 days. This regimen was well tolerated, produced trough SRL whole-blood levels >10 ng/mL at steady state, and inhibited whole-blood lymphocyte proliferation by >60% compared with pretreatment values. Therefore, 1.5 mg/kg SRL was chosen as the starting dose for transplant recipients.

Aortic Transplantation Study Design

A total of 12 NHPs underwent aortic transplantation. Six NHPs were untreated (controls) and 6 NHPs were treated with SRL from postoperative day 0 to 105, when the animals were euthanized. The oral liquid SRL solution (5 mg/mL; a gift from Dr Seghal, Wyeth Ayerst Co, Princeton, NJ) was delivered by gavage under ketamine (Ketaset) anesthesia. SRL was administered once daily at a dose of 1.5 mg/kg during the first week. Thereafter, SRL doses were
adjusted individually by assessing SRL blood concentrations, immune function, and clinical status.

Transplantation procedure, routine postoperative care, graft removal, and histology have been described previously. Intravascular Ultrasound Procedure

Intravascular ultrasound (IVUS) measurements were performed transfemorally on days 21, 42, 63, 84, and 105 after surgery. Sequential IVUS imaging from the native aorta and allograft segments was performed with a commercially available IVUS system (CardioVascular Imaging Systems/Boston Scientific Corp) with a 40-MHz, 2.6F mechanical ultrasound catheter (SciMed/Boston Scientific Corp). The imaging probe was positioned proximal to the graft and withdrawn at a constant speed of 0.5 mm/s with a motorized pullback device. The IVUS imaging run was recorded on videotape for analysis.

Offline 3D reconstruction of the IVUS data was performed with a system using EchoPlaque software (Indec Systems, Inc). Contour drawing of both the intima and lumen outer borders was performed manually at 1-mm intervals, and the interpolated contours for the remaining frames were generated automatically by the system. Areas and diameters for all lumen and intima contours were measured and volumes were automatically calculated by use of the following formula: \( \text{volume} = n \sum_{i=1}^{n} (A_i \cdot H) \), where \( A \) is the area of the lumen or intima outer border in a given cross-sectional image, \( H \) is the thickness of the slice (represented by a single tomographic IVUS image, 0.033 mm), and \( n \) is the number of IVUS images in the 3D data set. Volumetric (intimal volume [IV]) and cross-sectional (intimal area [IA]) parameters were measured for the middle 1 cm of the graft segments, as outlined in Figure 1. For each cross-sectional measurement, intimal index (II) was calculated as the percentage IA for each vessel area (II = 100 \( \times \) IA/VA) (Figure 2).

SRL Concentration

Whole-blood trough SRL concentrations were determined in whole-blood samples that were collected immediately before drug administration 3 times per week. Samples were analyzed by use of a modified high-performance liquid chromatography/electrospray-mass spectrometry method.

Pharmacodynamic Assays

Lymphocyte proliferation was quantified in whole blood by flow cytometric assessment of the percentage of proliferating cell nuclear antigen–positive cells in S,G2M phase. The assay was performed twice weekly in all NHPs. For calculation of drug effects, lymphocyte proliferation under immunosuppressive drug treatment was measured by a modified high-performance liquid chromatography/electrospray-mass spectrometry method.

Figure 1. IVUS measurements: volumetric analysis. IV was calculated for the middle 1-cm graft segments.

Figure 2. IVUS measurements: cross-sectional analysis. Cross-sectional IVUS image of an aortic allograft from an SRL-treated NHP on day 105. Vessel area (VA), lumen area (LA), IA (IA = VA – LA) were measured for each IVUS cross section. Little intimal thickening is visible.
expressed as percentage of pretreatment baseline values (ie, without immunosuppression). SRL doses were adapted individually to inhibit whole-blood lymphocyte proliferation by \( \geq 60\% \) compared with pretreatment values.

Statistical Analysis
All group data are reported as mean±SEM. STATA software for Windows (STATA Corp) was used to determine the levels of significance between different IVUS measurements. Within each group, changes in IA, IV, or II were compared by paired Student’s t test. Differences in IA, IV, and II between the 2 groups were analyzed with a 2-way ANOVA followed by a Tukey test for pairwise comparisons. Probability values of \( P<0.05 \) were considered to indicate statistical significance.

Results

Demographics
There was no difference between groups for weight mismatch between donor-recipient pairs, mixed lymphocyte reaction mismatch, and duration of graft ischemia.

Pharmacokinetics and Pharmacodynamics
In nontreated NHPs, mitogen-stimulated whole-blood lymphocyte proliferation was comparable to pretransplant values throughout the 105-day study period. In SRL-treated NHPs, the mean±SEM SRL plasma levels were 14.5±9 ng/mL, and lymphocyte proliferation was inhibited by \( 60\% \pm 5\% \) (mean±SEM) compared with pretransplant values (Figure 3). In 2 NHPs, the SRL dose was reduced to 1 mg/kg on days 21 and 24 because of diarrhea and weight loss. In another NHP, the SRL daily dose was increased on day 21 to 2 mg/kg because lymphocyte proliferation was insufficiently inhibited; thereafter, lymphocyte proliferation remained inhibited by \( \geq 60\% \) until day 105.

IVUS Measurements
The results of IVUS measurements are summarized in the Table and in Figures 4 through 6. The grafts in SRL-treated NHPs had significantly smaller IA, IV, and II compared with grafts from untreated NHPs at days 63, 84, and 105. Furthermore, in treated NHPs, IA, IV and II did not increase significantly from day 21 to day 105.

![Graph showing IA in control NHPs and NHPs treated with SRL.](Image)

**Figure 3.** Pharmacokinetics and pharmacodynamics. Inhibition of lymphocyte proliferation from untreated and SRL-treated NHP. Values are mean±SEM. PCNA indicates proliferating cell nuclear antigen.

**Figure 4.** IA of the middle 1-cm segment of aortic allografts. Graph showing IA in control NHPs and NHPs treated with SRL. Values represent mean±SEM. The probability value refers to the difference in IA between the 2 groups analyzed by 2-way ANOVA. *\( P<0.05 \), **\( P<0.01 \); post-hoc pairwise comparisons by Tukey test.

<table>
<thead>
<tr>
<th>IVUS Parameter/Postoperative Day</th>
<th>Nontreated Control NHPs</th>
<th>SRL-Treated NHPs</th>
<th>( P )</th>
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<tr>
<td>IA, mm(^2)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>21</td>
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<td>2.19±0.28</td>
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<td>42</td>
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</tr>
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<td>84</td>
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<td>2.67±0.55</td>
<td>&lt;0.01</td>
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<tr>
<td>105</td>
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<td>2.91±0.36</td>
<td>&lt;0.001</td>
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<tr>
<td>IV, mm(^2)</td>
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<td></td>
<td></td>
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<tr>
<td>21</td>
<td>24.9±1.23</td>
<td>21.6±3.04</td>
<td>NS</td>
</tr>
<tr>
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<td>25.3±2.09</td>
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<td>105</td>
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<tr>
<td>II, %</td>
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<tr>
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<td>13.3±1.7</td>
<td>NS</td>
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<tr>
<td>105</td>
<td>36.2±3.8</td>
<td>17.5±2.1</td>
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</table>

Values are mean±SEM.

Histology
Grafts from untreated NHPs showed the histological hallmarks of GVD along the entire length of each graft (Figure 7A). The GVD lesion consisted of concentric fibrointimal proliferation of spindle cells (SMCs and myofibroblasts) containing scattered macrophages and lymphocytes in a collagenous matrix in the intima. Unlike rat aortic allografts, the internal elastic membrane was preserved with regions of reduplication. The medial layer was obscured by fibrous tissue that extended into the surrounding adventitial and periadventitial soft tissues. Collections of mononuclear inflammatory cells were noted in the adventitia. The histology in these grafts was indistinguishable from human coronary allograft vasculopathy. In all treated NHPs, the allografts had minimal intimal thickening (Figure 7C).

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SMC α-actin staining confirmed that the intima in allografts of the untreated NHPs consisted of abundant numbers of SMCs (Figure 7B), whereas in the grafts from treated NHP, α-actin–positive cells were seen in the media only (Figure 7D).

**Laboratory Values**

Cholesterol levels were within the normal range throughout SRL treatment. Triglyceride levels remained in the normal range during the treatment period through day 84. The mean triglyceride level in the SRL-treated NHPs was significantly greater than the mean level in untreated NHPs only on day 105 (Figure 8).

**Discussion**

In the present study, we show that SRL monotherapy begun on the day of transplantation prevented the development of GVD.

GVD remains a major limitation to long-term graft survival. Conventional immunosuppressive agents fail to be effective in treatment or prevention of GVD, and reliance on rodent models of chronic rejection has limited further advances.

We describe a procedure that was the first to produce GVD in NHPs and in which the histopathology is indistinguishable from human allograft vasculopathy. This procedure produces GVD consistently in allografts over a period of 3 months, but GVD does not occur during this time in autografts. Furthermore, progression of allograft vasculopathy can be monitored by sequential IVUS examination of the grafts. Using this procedure, we have shown in previous studies that delayed administration of SRL halts progression of advanced lesions of GVD. In the present study, we used this procedure to determine whether SRL monotherapy prevents GVD.

The efficacy of SRL in preventing acute rejection has been related to trough drug levels, and our previously reported results suggested that the ability of SRL to halt progression of
GVD correlated with SRL blood levels. Furthermore, we have shown that inhibition of lymphocyte proliferation correlates highly with the immunosuppressive efficacy of studied drugs. Therefore, a preliminary study was conducted in 4 nontransplanted NHPs to evaluate the pharmacokinetics, pharmacodynamics, and tolerability of the starting dose of SRL intended to be used in transplant recipients. In the animals that received transplants, individual adjustment of the SRL dose maximized tolerability and ensured drug efficacy, since this individualized regimen produced desired trough SRL levels and inhibited lymphocyte proliferation by >60% compared with pretreatment values.

We show that SRL clearly prevents GVD, because IA and IV values for grafts from SRL-treated NHPs did not increase significantly between days 21 and 105. A significant difference in GVD could already be observed between SRL-treated and nontreated animals at day 63, and this difference became even greater on days 84 and 105.

Histology and immunohistochemistry of aortic allografts on postoperative day 105 clearly showed the efficacy of SRL in preventing GVD. Early after endothelial activation caused by alloimmune injury, mononuclear cells and α–actin–positive myofibroblasts are believed to migrate from the media to the intima, leading to the early intimal lesions of GVD. In our study, α–actin–positive SMCs were more evenly distributed in the media, and there were fewer positive cells in the intima in aortic allografts from SRL-treated NHPs than in graft from untreated NHPs. In the untreated NHPs, the high number of α–actin–positive cells in the intima suggested extensive migration of the SMCs from the media to the intima. These results are in concordance with in vitro studies showing that SRL inhibits SMC migration.

SRL was first shown to prolong allograft survival by 2 groups working independently. The number and severity of acute rejections correlate strongly with the incidence of GVD, and the efficacy of SRL for controlling acute rejection has been proved in various animal models and in clinical transplantation. Thus, our demonstration of the efficacy of SRL in preventing GVD may be at least partly because of its ability to prevent acute allograft rejection.

SRL acts by inhibiting cellular responses to cytokines and growth factors. Unlike calcineurin inhibitors (cyclosporine and tacrolimus), SRL inhibits the proliferation not only of immune but also of mesenchymal cells: SRL inhibits SMC proliferation in vitro and in vivo. SMC migration and proliferation is activated through multiple signaling pathways. SMCs leave their quiescent state and enter the cell cycle, associated with the induction of early-response genes. Cell division and growth are tightly controlled by a series of positive and negative regulators that act at sequential points through the cell cycle.

Recently, the tumor suppressor protein retinoblastoma protein (pRb) has been identified as a critical regulator of SMC proliferation. Phosphorylation and activation of pRb in response to mitogenic stimulation results in G/S transition and proliferation. The inhibition of pRb by pharmacological agents such as SRL results in cell cycle arrest in SMCs and inhibition of proliferation.

In vitro studies in fibroblasts have also shown that SRL blocks the antiapoptotic effect of the Gas6 transduction pathway. Thus, complete prevention of GVD in this study may also be a result of induction of apoptosis in vivo in the cellular components of GVD.

Study Limitations

This study is limited to observations in an experimental procedure of GVD, the relevance of which to clinical transplantation is still unknown. The effects of long-term SRL treatment on the prevention of GVD are unknown. Although it is possible that the hyperlipidemic side effects may reduce the advantage achieved by the antiproliferative effects of SRL, in SRL-treated patients, elevations in blood lipid levels decrease over time and are controlled by lipid-lowering therapy.

Conclusions

Our data provide evidence that SRL monotherapy effectively prevents the development of GVD at 3 months. Individual adjustment of the dose of SRL ensured drug efficacy, which may be because of the inhibition of both immune cell activation and SMC proliferation and migration. These and our previously reported results show that SRL not only halts progression GVD but also prevents GVD, thus suggesting the value of SRL for controlling GVD in clinical transplantation.

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References


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