Long-term effects of polymer-based, slow-release, sirolimus-eluting stents in a porcine coronary model

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Abstract

Background: Stent-based delivery of sirolimus (SRL) has shown reduction in neointimal hyperplasia and restenosis. The purpose of this study was to evaluate the chronic vascular response and the expression of cell cycle regulators after SRL-eluting stent implantation in a porcine coronary model. Methods: Forty-nine pigs underwent placement of 109 oversized stents (control, n = 54, SRL (140 μg/cm²), n = 55) in the coronary arteries with histologic analysis and Western blot (PCNA, p27kip1, CD45, MCP-1, IL-2, IL-6, TNF-β) at 3, 30, 90 or 180 days. Results: At 3 days, the mean thrombus area was similar for control (0.38 ± 0.19 mm²) and SRL (0.29 ± 0.09 mm²) stents. After 30 days, the mean neointimal area was significantly less for the SRL (1.40 ± 0.35 mm²) versus the control stents (2.94 ± 1.28 mm², p < 0.001). At 90 and 180 days, the mean neointimal area was similar for the SRL (3.03 ± 0.92 and 3.34 ± 0.99 mm²) as compared with control stents (3.45 ± 1.09 and 3.65 ± 1.23 mm²). Western blot analysis demonstrated an increased expression of p27kip1 in the vessel wall at 90 days for the SRL versus control stents (p = 0.05) but with increased levels of PCNA in the SRL as compared with control stents (p = 0.003). Conclusion: SRL-eluting stents favorably modulate neointimal formation for 30 days in the porcine coronary model. Long-term inhibition of neointimal hyperplasia is not sustained presumably due to delayed cellular proliferation despite increased levels of the cyclin-dependent kinase p27kip1 in the vessel wall.

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Keywords: Stents; Restenosis; Smooth muscle; Cell cycle

This article is referred to in the Editorial by A. Lafont (pages 575–576) in this issue.

We have previously documented that stent-based delivery of sirolimus (SRL) suppressed neointimal hyperplasia at 28 days presumably via inhibition of cell proliferation and the expression of inflammatory cytokines in the porcine coronary model [1]. These encouraging data provided the impetus to embark on long-term experimental studies to determine efficacy, biocompatibility as well as to assess for potential indicators of vascular toxicity such as medial necrosis, severe inflammation, aneurysm formation, delayed endothelialization, and stent thrombosis.

The purpose of the present study was to determine the long-term effects of SRL eluting stents on neointimal formation in the porcine coronary model. The temporal effects of SRL stents on neointimal formation and other components of arterial repair were assessed by qualitative and quantitative histopathology at 3, 30, 90 and 180 days. Additional experiments were conducted to measure biological markers of cell proliferation (PCNA), cell cycle activation (p27kip1), and inflammation (MCP-1, CD45, IL-2, IL-6, TNF-β) at 3, 30 and 90 days by Western...
blot analysis to determine the mechanism by which the SRL eluting stent inhibits neointimal formation.

1. Methods

1.1. Experimental studies

Stainless steel balloon expandable tubular stents (BX Velocity™ Cordis, Warren, NJ) were coated with a thin layer of a co-polymer containing \( \approx 140 \mu \text{g/cm}^2 \) of SRL (Wyeth-Ayerst, Princeton, NJ) a slow (Cypher™ Cordis) release delivery system [1]. Drug elution is >90% complete after 12 weeks for this drug eluting system. Bare metal BX Velocity™ stents served as controls. All stents were individually packaged, coded with a serial number on the packaging label and ETO sterilized. The identity of each serial number was known only by the sponsor to permit deployment and analysis of each stent in a blinded fashion.

Experimental studies were conducted after IACUC approval in accordance with NIH and AHA guidelines for animal research. Forty-nine (\( \approx 50 \) to 75 kg) pigs (34 Yucatan mini-pigs, 79 stents for histopathology analysis; 15 Juvenile Yorkshire pigs, 30 stents for Western Blot analysis) underwent placement of 109 stents (control, \( n = 54 \), SRL, \( n = 55 \)) in the left anterior descending, circumflex or right coronary artery. The guiding catheter was used as a reference in order to obtain a 1.1–1.3:1 stent to artery ratio as compared with the baseline vessel diameter. Animals were allowed to recover and returned to care facilities where they received a normal diet, aspirin 325 mg daily for the duration of the study and clopidogrel 75 mg daily for 2 months. At 3 days (\( n = 10 \)), 30 days (\( n = 13 \)), 90 days (\( n = 16 \)), or 180 days (\( n = 10 \)) the animals were euthanized after completion of coronary angiography to obtain specimens for histological analysis or Western blot of stented arterial segments.

1.2. Quantitative coronary angiography

Angiographic images of stent implants for histological analysis (\( n = 79 \)) were saved to a CD-ROM disk in a standard DICOM format. Images were analyzed on a PC-based quantitative coronary angiographic analysis software program (CCAL, Stanford University Medical Center, Stanford, CA). The guiding catheter served as a reference for calibration for all measurements. Measurements included: baseline reference vessel diameter, balloon inflated diameter, post-stent minimal lumen diameter, follow-up reference vessel diameter, follow-up minimal lumen diameter, follow-up percent diameter stenosis. The balloon to artery ratio was calculated as: the balloon inflated diameter/reference vessel diameter. The percent diameter stenosis was calculated as: 100 \times [1 – (minimal lumen diameter/reference vessel diameter)].

1.3. Biological markers of inflammation and cell cycle activation

At 3, 30 or 90 days, SRL (\( n = 15 \)) and control (\( n = 15 \)) stent segments were removed from freshly isolated arterial specimens. The excess or loose perivascular tissue was carefully dissected from the stent. The vessel was bisected to allow extraction of the stent from the vessel wall. Tissue samples were then snap frozen in liquid nitrogen and stored at \(-70 \, ^\circ \text{C} \). Vessel wall expression of PCNA (DAKO: M0879), p27kip1 (Santa Cruz Biotechnology: sc-528), MCP-1 (R&D Systems: MAB679, clone 23007.111), TNF-\( \beta \) (Boehringer Mannheim: 1141333, clone 9B9), CD45, IL-2 and IL-6 (R&D Systems: MAB114; AF652; AF686) was evaluated by Western blot analysis. Briefly, protein extracts (50 \mu\text{g}) were size fractionated on SDS-polyacrylamide gels, transferred to nitrocellulose membrane. Positive control for each target was run on the same gel. Membranes were incubated with an affinity purified polyclonal antibody to PCNA, p27kip1, MCP-1, TNF-\( \beta \), CD45, IL-2 and IL-6 respectively, washed and incubated with secondary antibody. Signals were detected by the ECL chemiluminescence detection system. Autoradiographic signals were quantified by densitometry. Tubulin was used as an internal control to ensure equal amount of protein extract in each sample. All results were compared to the aorta of the respective animal. SRL levels in the arterial wall, and the stent were determined at 90 days using HPLC [2].

1.4. Pathologic evaluation

Immediately following euthanasia, the hearts were harvested, and the coronary arteries were perfusion-fixed with 10% buffered formalin at 100 mm Hg. The stented coronary artery segments were processed for plastic embedding, staining and morphometric analysis of six sections from the proximal through the distal margin of the stent [1,3,4]. The specimens were embedded in methyl methacrylate and sections were obtained with a Beuhler isomet saw (Beuhler, Evanston, IL). The sections were then polished, mounted on a glass slide and stained with metachromatic stain. All histopathologic analysis was completed by a single independent investigator (F.T.) blinded to treatment group. Vessel morphometry (SigmaScan Morphometric Software, Jandel Scientific, San Rafael, CA) and morphologic analysis of injury, inflammation, endothelialization, fibrin, and smooth muscle content were completed using published methods [1,3,4].

Stent endothelialization score was defined as the extent of the circumference of the arterial lumen covered by endothelial cells and graded from 1 to 3 (1 = 25%, 2 = 25% to 75%, 3 = >75%). Injury score was determined by the method of Schwartz et al. [3]. Inflammation was graded as 0, none; 1, scattered inflammatory cells; 2, inflammatory cells encompassing 50% of a strut in at least 25% to 50% of the
circumference of the artery; 3, inflammatory cells surrounding a strut in at least 25% to 50% of the circumference of the artery. The intimal fibrin content was graded as 0, no residual fibrin; 1, focal regions of residual fibrin involving any portion of the artery or moderate fibrin deposition adjacent to the strut involving <25% of the circumference of the artery; 2, moderate fibrin involving >25% of the circumference of the artery or heavy deposition involving <25% of the circumference of the artery; or 3, heavy fibrin deposition involving >25% of the circumference of the artery. The intimal SMC content was scored as 1, sparse SMC density involving any portion of the artery and for moderate SMC infiltration less than the full thickness of the neointima involving <25% of the circumference of the artery; 2, moderate SMC infiltration less than the full thickness of the neointima involving >25% of the circumference of the artery; or 3, dense SMC content the full thickness of the neointima involving >25% of the circumference of the artery. A positive giant cell reaction was defined as the presence of giant cells on a single section from the stent.

1.5. Statistical analysis

The morphometric measurements from each of the 4-stent sections were summed and divided by 4 to generate the mean value for each parameter within the stent. For continuous variables, such as morphometric parameters, the mean differences between treatment groups were tested with ANOVA. For morphologic parameters, scores were assigned to each of the four sections within the stented segment, the median value used as the score for the stent. The data were ranked within each cohort (3, 30, 90, or 180 days) and stratified. An ANOVA was performed on these ranks. Categorical data were compared with chi-square analysis. Data are expressed as mean ± S.D. unless otherwise stated. All statistical analysis was performed with SAS® system software.

2. Results

A total of 109 of 109 stents were successfully implanted in the coronary arteries of 49 swine. Stent migration occurred in one implant during balloon withdrawal (SRL group) that necessitated post-dilation with a 4.0-mm diameter, 20-mm-long non-compliant balloon. A total of 49 of 49 animals (100%) survived the intended study interval without clinical or angiographic stent thrombosis. The animals remained well throughout the study without abnormal temperature, weight loss, or other major health problems.

2.1. Quantitative coronary angiography

The baseline vessel diameter was similar for both SRL and control stents (range 2.55–2.94 mm). The balloon to artery ratio was similar for each group, approximately 1.2 to 1 (range 1.16–1.29 to 1). After 30 days, the SRL group had significantly less in-stent %stenosis (−24.4 ± 17.7%) versus the control stents (−3.6 ± 10.5%, p < 0.05). At 90 and 180 days, the control (90 days, 8.7 ± 8.5; 180 days, 3.9 ± 11.4) and SRL (90 days, 2.5 ± 16.5; 180 days, 0.8 ± 9.1) stents each exhibited minimal and similar angiographic narrowing. There were no cases of greater than 50% diameter stenosis for the SRL or control stents at 3, 30, 90 or 180 days. Qualitative analysis of angiograms failed to identify intraluminal filling defects, edge effects or aneurysms for control or the SRL groups.

2.2. Histology

The histomorphometry and a semi-quantitative scoring for injury, inflammation and intimal fibrin content at 3, 30, 90 and 180 days for control and the SRL eluting stents are summarized in Tables 1–3 and Figs. 1–3. Vessel morphology of proximal and distal adjacent non-stented sections were similar for each group at all time points (data not shown). At 3 days, SRL and control stents had a similar appearance with fibrin-platelet deposition and acute inflammatory cells (PMNs) (Fig. 3). After 30 days, a significant (50%) reduction in neointimal area was observed for SRL stents versus control stents (Table 1, Figs. 1 and 2). The reduction in neointimal area for SRL stents resulted in 50% less cross-sectional area narrowing in comparison with control stents. The neointima for the SRL stents contained SMC, matrix proteoglycans and regions of residual fibrin deposition (Fig. 3). In frequent regions of acellular plasma-

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Stent/IEL area</th>
<th>Neointimal area</th>
<th>% area stenosis</th>
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<tbody>
<tr>
<td><strong>3 days</strong></td>
<td></td>
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</tr>
<tr>
<td>Control (n=10)</td>
<td>8.31 ± 0.59</td>
<td>0.38 ± 0.19</td>
<td>4.6 ± 2.3</td>
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<tr>
<td>SRL (n=10)</td>
<td>8.61 ± 0.45</td>
<td>0.29 ± 0.09</td>
<td>3.4 ± 0.9</td>
</tr>
<tr>
<td><strong>30 days</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control (n=9)</td>
<td>8.48 ± 0.41</td>
<td>2.94 ± 1.28</td>
<td>34.8 ± 15.3</td>
</tr>
<tr>
<td>SRL (n=10)</td>
<td>8.23 ± 0.80</td>
<td>1.40 ± 0.35**</td>
<td>17.0 ± 4.33†</td>
</tr>
<tr>
<td><strong>90 days</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=10)</td>
<td>8.24 ± 1.45</td>
<td>3.45 ± 0.22</td>
<td>42.5 ± 14.9</td>
</tr>
<tr>
<td>SRL (n=10)</td>
<td>8.90 ± 1.60</td>
<td>3.03 ± 0.92</td>
<td>35.5 ± 13.5</td>
</tr>
<tr>
<td><strong>180 days</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=10)</td>
<td>8.15 ± 0.69</td>
<td>3.65 ± 1.23</td>
<td>45.3 ± 16.5</td>
</tr>
<tr>
<td>SRL (n=10)</td>
<td>8.27 ± 0.95</td>
<td>3.34 ± 0.99</td>
<td>42.2 ± 18.1</td>
</tr>
</tbody>
</table>

The neointimal area reported at 3 days represents the area of thrombus measured within the stent.

* p = 0.0019 versus 30-day control.
† p ≤ 0.0001 versus 90- and 180-day SRL.
‡ p = 0.0025 versus 30-day control.
§ p ≤ 0.015 90- and 180-day SRL.
like collections were uniquely observed in the SRL stents. The SMC content was less for the SRL stents as compared with the control stents at 30 days \((p = 0.01)\). The media appeared intact with localized regions of compression in areas of strut-induced vessel injury. Medial necrosis was not observed in any sections from SRL or control stents. Endothelialization scores were identical (>75% complete) for SRL and control stents.

After 90 and 180 days, the mean neointimal area and %area stenosis were similar for SRL and control stents (Table 1 and Fig. 2). At 90 days, the neointima for the SRL stents contained SMC, matrix proteoglycans and regions of residual fibrin deposition (Fig. 3). Localized regions of acellular plasma-like collections, evident at 30 and 90 days, were no longer observed at 180 days in the SRL stent sections. Strut associated fibrin was more prevalent at 90 days for SRL versus control stents \((p = 0.02)\). At 180 days, strut-associated fibrin was not observed for SRL or control stents. SMC content score was similar for the SRL stents as compared with the control stents at 90 and 180 days. Medial necrosis was not observed in any sections from SRL or control stents. Inflammatory cells, predominantly lymphocytes, were observed in areas adjacent to stent struts for both SRL and control stents. Eosinophils were not observed in any sections from SRL or bare metal stents. At 90 days, a giant cell reaction was evident in 3 of 10 stents in the SRL and in 1 of 10 control stents \((p = 0.47)\). At 180 days, a giant cell reaction was evident in at least one section from 5 of 10 stents in the SRL and in 2 of 10 control stents \((p = 0.23)\). Endothelialization scores were identical, >75% complete, for SRL and control stents at both 90 and 180 days.

### 2.3. Western blots

The mean arterial tissue content of SRL was 0.32 ± 0.24 ng/mg arterial tissue at 90 days. PCNA and p27kip1 expression for control and SRL stents at 3, 30 and 90 days are demonstrated in Fig. 4. Western blot analysis failed to detect increased levels of expression of MCP-1, CD45, IL-2, IL-6, and TNF-β above normal non-injured sections of the aorta.

### 3. Discussion

The present study documents the temporal vascular response for SRL-eluting stents to 180 days in the porcine coronary model. The SRL stent effectively suppresses neointimal formation for the first 30 days in comparison with bare metal stents. Late neointimal formation occurs

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**Table 2**

<table>
<thead>
<tr>
<th>Group</th>
<th>Grade 0</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>(p) value</th>
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</thead>
<tbody>
<tr>
<td>3 days Control ((n=10))</td>
<td>50% (5/10)</td>
<td>20% (2/10)</td>
<td>30% (3/10)</td>
<td>0% (0/10)</td>
<td>0.15</td>
</tr>
<tr>
<td>SRL ((n=10))</td>
<td>30% (3/10)</td>
<td>10% (1/10)</td>
<td>60% (6/10)</td>
<td>0% (0/10)</td>
<td></td>
</tr>
<tr>
<td>30 days Control ((n=9))</td>
<td>33% (3/9)</td>
<td>22% (2/9)</td>
<td>45% (4/9)</td>
<td>0% (0/10)</td>
<td>0.49</td>
</tr>
<tr>
<td>SRL ((n=10))</td>
<td>20% (2/10)</td>
<td>20% (2/10)</td>
<td>60% (6/10)</td>
<td>0% (0/10)</td>
<td></td>
</tr>
<tr>
<td>90 days Control ((n=10))</td>
<td>40% (4/10)</td>
<td>10% (1/10)</td>
<td>30% (3/10)</td>
<td>20% (2/10)</td>
<td>0.56</td>
</tr>
<tr>
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<td>20% (2/10)</td>
<td>10% (1/10)</td>
<td>60% (6/10)</td>
<td>10% (1/10)</td>
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<tr>
<td>180 days Control ((n=10))</td>
<td>30% (3/10)</td>
<td>10% (1/10)</td>
<td>50% (5/10)</td>
<td>10% (1/10)</td>
<td>0.06</td>
</tr>
<tr>
<td>SRL ((n=10))</td>
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<td>0% (0/10)</td>
<td>40% (4/10)</td>
<td>50% (5/10)</td>
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**Table 3**

<table>
<thead>
<tr>
<th>Injury in SRL and control stents by time</th>
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<tr>
<td>Group</td>
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<tr>
<td>-------</td>
</tr>
<tr>
<td>3 days Control ((n=10))</td>
</tr>
<tr>
<td>SRL ((n=10))</td>
</tr>
<tr>
<td>30 days Control ((n=9))</td>
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<td>SRL ((n=10))</td>
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<tr>
<td>90 days Control ((n=10))</td>
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<tr>
<td>SRL ((n=10))</td>
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<tr>
<td>180 days Control ((n=10))</td>
</tr>
<tr>
<td>SRL ((n=10))</td>
</tr>
</tbody>
</table>
between 30 and 90 days for SRL stents at least in part due to inflammation and cellular proliferation. Angiographic and histological data documents a similar minimal degree of angiographic %diameter obstruction and mean neointimal area for the SRL and bare metal stents at 90 and 180 days. These observational data failed to identify medial necrosis, aneurysm formation, or excessive thrombosis for the SRL stents. Together, these data provide additional insights into the mechanism and efficacy of the SRL-eluting stent in normal porcine coronary arteries while raising questions regarding the potential durability of this drug eluting vascular prosthesis.

3.1. Experimental models of restenosis and drug-eluting stents

Previous studies have documented efficacy of SRL-eluting stents in two experimental models at 30 days [1,2]. In addition, these studies also supported evidence of polymer biocompatibility in two species at 60 days after...
An objective of the present study was to validate prior experimental observations and to characterize the chronic effects of the SRL-eluting stent on neointimal formation, regulatory proteins of the cell cycle and expression of inflammatory cytokines in a porcine coronary model.

In the porcine coronary model, studies employ oversized stent placement, typically 10% to 30% greater than the baseline vessel dimensions, to induce injury and neointimal formation. Over a decade has passed since Schwartz et al. [3] demonstrated the strong relationship between stent-induced vessel injury and neointimal formation at 28 days in the porcine coronary model. Subsequent studies by Kornowski et al. [4] and Welt and Rogers [5] documented the interactions of vessel wall injury and inflammation in contributing to neointimal formation at 28 days. Unfortunately, only limited published data exists to characterize long-term response to stenting in this model. Should we expect the treatment effect with SRL-eluting stents to persist beyond 30 days in the porcine coronary model?

In the present study, the mean neointimal area was approximately 50% less for the SRL versus the control stents at 30 days. In contrast, by 90 days, the mean neointimal area was similar for the SRL stents (≈ 3.00 mm²), as compared with control stents resulting in similar percent area in-stent stenosis. Light microscopy and SEM (unpublished data on file) of the SRL stents documented complete coverage of the luminal surface with endothelial cells within 30 days. Our data document detection of SRL (0.32 ng/mg) in the arterial tissue at 90 days with evidence of increased levels of p27kip1, a mediator of the antiproliferative effects for this compound, in the vessel wall [6]. The expression of PCNA, a marker of cell proliferation, was more abundant in the vessel wall after 30 days for SRL eluting stents in contrast to control stents. These data suggest that other cell cycle regulators could also participate in SRL-mediated in vivo inhibition of SMC proliferation, the possibility of an insufficient arterial drug level at 90 days, or perhaps the presence of other potent sustained physiologic stimuli of SMC proliferation and neointimal formation not sufficiently affected by SRL-mediated inhibition of the cell cycle [7].

Histological data documents a progressive increase in injury and inflammation scores between 30 and 180 days for the SRL as compared with control stents. This observed
3.2. Comparison with clinical data for sirolimus-eluting stents

The safety and feasibility of the SRL-eluting stent were evaluated in a 45 patient phase I clinical trial and documented a stable in-stent MLD for the SRL-eluting stents after 2 years [8,9]. The RAVEL and SIRIUS randomized clinical trials have documented a significant reduction in clinical and angiographic restenosis at 12 months for SRL-eluting versus the BX Velocity stent in patients with focal de novo native coronary arterial lesions [10–13]. Recent data from the RAVEL trial have revealed a significantly lower frequency of target vessel revascularization at 3 years for SRL-eluting versus bare metal stents, despite four cases of target vessel revascularization between 1 and 3 years in the SRL group [13].

The clinical efficacy of SRL-eluting stents would not be expected based on the degree and duration of suppression of neointimal formation documented in normal porcine coronary arteries. The vastly different pharmacodynamics of SRL-eluting stents observed to date in human clinical trials versus preclinical models may be attributed to differences in species response to SRL, anatomic substrate and physiological stimulus for neointimal formation. We have previously reported and confirm in the present study a 50% reduction for SRL stents in comparison with control stents at 30 days in the porcine coronary model [1]. In the present study, the treatment effect for SRL stents was not observed beyond 30 days. In contrast, two randomized clinical trials have demonstrated a >90% inhibition of neointimal formation for SRL stents in comparison with bare metal stents as measured by volumetric IVUS after 6 months [7–9]. A precise...
explanation for the discrepancy between preclinical and clinical results with SRL eluting stents remains elusive.

Wright et al. [14] have documented a two-fold difference in mitogen-stimulated peripheral blood mononuclear cells and mixed lymphocyte response for porcine versus human cells exposed to similar concentrations of SRL. A distinct species response to the antiproliferative and immunosuppressive effects of SRL may account in part for the disparity between 30-day porcine and 6-month human clinical data for this drug eluting stent. The vastly different anatomic and cellular substrate of atherosclerotic human versus normal porcine coronary arteries could also account for this dose response discrepancy. Zohlnhofer et al. [15] have demonstrated a higher prevalence of the FKBP-12 binding protein, the intracellular receptor for SRL, in intimal derived versus medial smooth muscle cells. Thus, perhaps a more abundant expression of the FKBP-12 receptor in atheroclerotic human coronary arteries in comparison with normal porcine coronary arteries enhances the efficacy of stent-based delivery of SRL in man. The differences in physiologic stimuli for neointimal formation are of obvious importance when comparing diseased human coronary arteries to normal porcine coronary arteries.

4. Conclusions

SRL-eluting stents favorably modulate neointimal formation for 30 days in the porcine coronary model. Long-term inhibition of neointimal hyperplasia was not sustained presumably due to delayed cellular proliferation, despite increased expression of the cyclin-dependent kinase p27Kip1. Our data highlight the necessity to improve our understanding of preclinical cardiovascular drug and device testing as well as to explore refinements of stent-based drug delivery. Randomized clinical trials with 3 to 5 years observation are necessary to document a sustained benefit for drug eluting stents.

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