Evaluation of Polymer-Based Comparator Drug-Eluting Stents Using a Rabbit Model of Iliac Artery Atherosclerosis

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Background—Although atherosclerotic models, especially in the rabbit, have existed for a long time, a comparative study of various drug-eluting stent (DES) implantations in atherosclerotic arteries have not been systematically studied.

Methods and Results—New Zealand White rabbits (n=44) with induced atheroma received bilateral iliac artery stents: bare metal stent (BMS) (Driver) or a stent eluting zotarolimus (ZES) (Endeavor), sirolimus (SES) (Cypher), or everolimus (EES) (Xience V). After 28 days, tissues were harvested for histomorphometric analyses, en face analysis of endothelial coverage, and expression of endothelial nitric oxide synthase (eNOS). Area measurements of external elastic lamina and stent area were similar. Neointimal area was significantly less in all DES versus BMS, which was least in SES and EES; similar trends were noted for cell proliferation. Uncovered struts were greater for SES and EES and least in BMS, whereas ZES were in between and associated with the least fibrin. Macrophages of the neointima were significantly less for all DES relative to BMS. Plaque calcification underneath stents, however, was significantly greater in SES and ZES than in BMS. Although endothelial coverage in between struts was comparable between BMS and DES, there was significantly greater expression of eNOS in BMS and ZES relative to SES and EES.

Conclusions—The rabbit atherosclerotic model of stenting showed delayed healing and significantly greater reduction of neointima following implantation of SES and EES; however, delayed healing was less in ZES with greater neointima (but less than BMS), endothelial regrowth, and eNOS expression. (Circ Cardiovasc Interv. 2011;4:38-46.)

Key Words: atherosclerosis ■ stents ■ models animal ■ drug-eluting stents

Randomized clinical trials of patients receiving drug-eluting stents (DES) demonstrate significantly fewer adverse outcomes than for equivalent patients receiving bare metal stents (BMS).1 Despite the major technological advances in stent therapy over the past 2 decades, restenosis and thrombosis (primarily late and very late) remain principal factors that weigh on the morbidity and mortality associated with these devices.2,3 Although the currently available drugs loaded on stents have proven to be remarkably effective for suppression of smooth muscle cell proliferation and inflammation, these agents have a profound impact on vascular healing identified by persistent fibrin and impaired restoration of endothelial coverage and function, which may contribute to complications of thrombosis.4

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In addition to design and mode of drug delivery and release, the impact of the underlying disease (plaque milieu) is likely involved in arterial healing. Pathological studies of stent behavior in diseased vessels, however, are derived mostly from autopsy data, where translational animal models capable of foreseeing biological responses and outcomes of DES in humans are lacking. Therefore, a broader availability of relevant animal models further characterized by appropriate histological measures for evaluation of safety and efficacy should improve the preclinical assessment of DES performance before launching into human clinical trials.5

Although arterial repair clearly is more rapid in animals than in man, the sequence of biological events that dictate vascular healing share many similarities.6 Preclinical assessment of DES classically involves the porcine coronary artery, where a recently issued consensus report by Schwartz et al7 recommended that safety evaluations for stents be performed in their intended vascular beds. The generalized use of juvenile animals with normal vessel morphology, however, may not necessarily best reflect the condition of the patients with coronary artery disease for whom these stents are intended. Thus, various groups are beginning to recognize the importance of device testing in perhaps a more appropriate atherosclerotic environment in the hope of achieving a clearer profile of potential clinical outcomes.5,8

There are recognized advantages and disadvantages between small and large animals amenable to interventional...
procedures. The potential value of the swine coronary stent model for confirmation of device safety, as classically accepted, may in fact be compromised by accelerated rates of healing because this is uncharacteristic of humans. Moreover, the swine often shows higher-than-expected inflammation or granulomatous reactions, also unlike the experience with human stents. On the contrary, despite the absence of an “appropriate” vascular bed, there are clear advantages to the rabbit iliofemoral model, which inherently exhibits less of an inflammatory foreign body reaction and slower rates of endothelial strut coverage, although the latter process still remains markedly accelerated relative to human.

Another major concern of stent evaluation in normal arteries has been the failure to demonstrate long-term efficacy, which compromises the assessment of device performance and continues to limit our understanding of the pathophysiology of restenosis. In this regard, atherosclerotic models of stenting not only may support efficacy and safety studies, but also could provide a better translation bridge toward further improving DES technology. Although a porcine model of accelerated atherosclerosis is available, to our knowledge, there are no reports of its use with stents. In the present study, the healing characteristics of leading comparator DES were assessed in a rabbit atherosclerotic model with outcomes specific to neointimal growth and composition, re-endothelialization, and proteins specific to endothelial function with the intent to identify similarities to DES implantations in humans.

Methods

The study protocol was reviewed and approved by the Institutional Animal Care and Research Committee, Jack H. Skirball Center for Cardiovascular Research (Orangeburg, NY). Experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Induction of Atherosclerosis

Before deploying stents, iliofemoral artery lesions in hypercholesterolemic rabbits were produced as previously described. Briefly, male New Zealand White rabbits were fed an atherogenic diet consisting of 1% cholesterol and 6% peanut oil for 5 weeks. One week after high-cholesterol feeding, iliac arteries were injured with a 3-F Fogarty balloon. The balloon injury focally accelerates atheroma formation that is generally smooth muscle rich with lipid-laden macrophages more closely resembling so-called human fibroatheroma than typical foam cell lesions produced by hypercholesterolemia alone. After arterial injury, animals were continued on an atherogenic diet for another 4 weeks and then switched to a diet lower in cholesterol (0.025%) for the remainder of the study (ie, 4 weeks after stent implantation) (Figure 1).

Stent Placement

Animals were assigned randomly to receive 90 stents consisting of either a BMS (Driver; Medtronic CardioVascular; Santa Rosa, CA) (n=30) or one of the following DES: (1) zotarolimus rapid-release (ZES) (Endeavor [not slow-release Endeavor Resolute]; Medtronic CardioVascular) (n=20), (2) sirolimus (SES) (CYPHER; Cordis Corporation; Warren, NJ) (n=20), and (3) everolimus (EES) ( XIENCE V; Abbott Vascular; Santa Clara, CA) (n=20). The stents were implanted in bilateral iliac arteries. All stents sizes were 3 mm × 18 mm. The operator was blinded to the treatment randomization up until the time of implantation. (For details, see supplemental Methods.)

Total Plasma Cholesterol

Rabbits were phlebotomized for measurement of total plasma cholesterol at days 0 (baseline), 7 (1% cholesterol and balloon injury), 35 (cholesterol diet switch to 0.025%), 63 (stent implantation), and 91 (stent harvest) (Figure 1).

Stent Harvest and Preparation

Stents were harvested at 28 days after implantation and then processed for (1) light microscopy, (2) scanning electron microscopy (SEM) for en face evaluation of endothelial coverage, and (3) expression of endothelial nitric oxide (eNOS) by immunofluorescent staining and confocal microscopy. For evaluation of endothelial surfaces, the stents were first bisected longitudinally to expose the luminal surface where one half was designated for SEM and the opposite for immunohistochemical analysis. (For details, see supplemental Methods.)

Light Microscopy and Histomorphometry

Specimens for light microscopy were embedded in methylmethacrylate resin, segmented at 2- to 3-mm intervals, sectioned, and stained by hematoxylin-eosin or modified Movat pentachrome as previously described. Additional sections from the midportion of the stent were held in reserve for Carstairs staining for fibrin and immunohistochemical identification of macrophages (RAM11) and cell proliferation by an anti-bromodeoxyuridine (BrdU) antibody. Histological sections were measured using computer-assist software (IPLab; Scannalytics, BD Biosciences; San Jose, CA). Cross-sectional area measurements included the external elastic lamina, stent, and lumen. A mean neointimal thickness for each section was determined over and between struts, defined as the distance at the inner strut surface to the luminal border and internal elastic lamina to the luminal surface, respectively. In addition to area and thickness measurements, stented segments were assessed for the presence of uncovered struts, fibrin, inflammation, cholesterol clefts, and calcification. (For details, see supplemental Methods.)

SEM

Composites of serial en face SEM images were acquired at low power (×15 magnification) and digitally assembled to provide a
complete view of the entire luminal stent surface. The images were further enlarged (×200 magnification), allowing direct visualization of endothelial cells. The extent of endothelial surface coverage above and between stent struts was traced from acquired images by morphometry software (IPLab). The results are expressed as the percentage of total surface area above or between struts with endothelial coverage.21 (For details, see supplemental Methods.)

**Immunohistochemical Detection of eNOS**

Low-power digital images of the luminal surface were acquired systematically using a ×10 objective from regions of interest involving equally spaced proximal, middle, and distal regions between stent struts and digitally quantified using color threshold methods (IPLab). The quantified data for eNOS was then expressed as percentage of total area. Negative and positive control staining was represented by omission of the primary antibody and nonstented segments remote from the stent. (For details, see supplemental Methods.)

**Statistical Analysis**

All values were expressed as mean±SD. Normality of distribution was tested with the Wilk-Shapiro test. Statistical comparisons of normally distributed measurements between stent groups were performed by linear generalized estimating equation (GEE) modeling with an assumed Gaussian distribution, an identity link function, and an assumed exchangeable structure for the within-cluster correlation matrix. GEE was necessary because of the clustered nature of >1 individual lesion measured from 1 animal, resulting in unknown correlations among measurements within these lesion clusters. Logistic GEE with an assumed binomial family distribution, a logistic link function, and an exchangeable structure in the correlation matrix were used to assess the presence of cholesterol clefts in underlying plaque. Comparison of each pair of stents was based on the estimated marginal means with sequential Bonferroni correction. For the comparison of nonnormally distributed data (fibrin score, uncovered struts, BrdU-positive cell, endothelial coverage by SEM), Kruskal-Wallis test was used followed by post hoc analysis using a Wilcoxon rank sum test with sequential Bonferroni correction to assess significant differences between each pair. A \( P < 0.05 \) was considered statistically significant. All statistics were calculated with SPSS version 19 (SPSS Inc; Chicago, IL) software.

**Results**

**Animal Condition and Cholesterol Levels**

Two animals died before stent deployment. One animal was anorexic, lethargic, and icteric and died at 8 weeks after initiation of the atherosclerotic diet. The second animal was found dead at 6 weeks before stent implantation; the cause of death in this animal could not be confirmed. During stent implantation, 1 animal was found to have bilateral angio-graphic occlusions of the iliac arteries, and 1 was found to have unilateral occlusion in left iliac artery; these arteries were excluded from the implantation matrix. Eighty-seven stents were harvested from 44 animals that appeared to be in generally good health without overt signs of liver toxicity. Of the 87 stents, 8 (2 BMS, 2 ZES, 2 EES, 2 SES) were used for preliminary studies involving *en face* immunostaining of CD31, which were excluded from the main analysis because of too few stents in each group; therefore, a total of 79 stents were included in this report (supplemental Table 1).

Mean levels of circulating cholesterol at baseline were 44±11 mg/dL, which increased after initiation of the 1% cholesterol diet until the diet switch to 0.025% cholesterol at 35 days (1832±700 mg/dL). At the time of stenting (63 days) and termination of the study (91 days), the serum cholesterol levels were markedly reduced (846±429 mg/dL and 1061±616 mg/dL, respectively).

**Morphometric Analysis**

Stent patency was confirmed by angiography, and ex vivo radiographic imaging showed no evidence of thrombosis or strut fracture in any of the devices. Area measurements of external elastic lamina and stent size were similar between BMS and comparator DES. On the contrary, BMS significantly differed from all DES, particularly with regard to neointimal area (BMS, 1.65±0.43 mm²; ZES, 1.15±0.28 mm²; EES, 0.70±0.17 mm²; SES, 0.94±0.23 mm²) (Table). Similarly, percent stenosis and neointimal thickness were reduced in DES; however, ZES did not achieve a statistical difference compared to BMS, whereas EES and SES were significantly different (Table). Further, the percentage of uncovered struts was significantly greater in all DES than in BMS; however, SES exhibited a significantly greater percentage of uncovered struts than ZES (SES, 64.58±29.27%; ZES, 30.45±19.01%; \( P = 0.044 \)) (Table, Figure 2). The underlying plaque area was greatest for BMS and ZES and similarly least for SES compared to BMS, whereas EES was significantly different (Table). All values were expressed as mean±SD. EEL indicates external elastic lamina.

**Table. Morphometric Analysis of 28-Day BMS and Comparator DES Implanted in the Atherosclerotic Iliac Artery of Rabbit**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BMS (n=10)</th>
<th>ZES (n=10)</th>
<th>EES (n=10)</th>
<th>SES (n=9)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEL area, mm²</td>
<td>7.64±0.87</td>
<td>7.59±1.12</td>
<td>6.98±0.72</td>
<td>7.05±0.56</td>
<td>0.30</td>
</tr>
<tr>
<td>Plaque area, mm²</td>
<td>1.35±0.43</td>
<td>1.17±0.19</td>
<td>0.82±0.61</td>
<td>0.83±0.24</td>
<td>0.048</td>
</tr>
<tr>
<td>Stent area, mm²</td>
<td>5.88±0.71</td>
<td>5.68±0.82</td>
<td>5.58±0.56</td>
<td>5.58±0.36</td>
<td>0.73</td>
</tr>
<tr>
<td>Neointimal area, mm²</td>
<td>1.65±0.43</td>
<td>1.15±0.28*</td>
<td>0.70±0.17†</td>
<td>0.94±0.23*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Area of stenosis, %</td>
<td>28.43±8.13</td>
<td>20.95±4.07</td>
<td>12.82±2.12‡</td>
<td>16.85±4.54*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Neointimal thickness, mm</td>
<td>0.23±0.06</td>
<td>0.17±0.05</td>
<td>0.11±0.02‡</td>
<td>0.11±0.05*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Uncovered struts, %</td>
<td>1.70±2.26</td>
<td>30.45±19.01*</td>
<td>44.16±21.92*</td>
<td>64.58±29.27†</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD. EEL indicates external elastic lamina.

*Significantly different from BMS.
†Significantly different from BMS and ZES.
significantly higher for ZES and SES than for BMS \((P<0.001)\). Interestingly, the percent area of neointimal macrophages was significantly less for all comparator DES, highest for BMS \((P<0.001)\), and of borderline significance for the underlying plaque \((P=0.08)\).

Neointimal Healing

Neointimal fibrin scores at 28 days were considered low for all stents, although ZES showed the least accumulated fibrin among comparator DES (Figure 3). There was greater fibrin deposition in SES and EES than in BMS and ZES; however, the difference did not achieve significance \((BMS, 0.06±0.14; ZES, 0.07±0.14; EES, 0.27±0.26; SES, 0.41±0.43; P=0.08)\). Similar trends also were noted for the percentages of total struts with fibrin, with significantly lower values for BMS and ZES versus EES and SES \((P<0.001)\). For morphological parameters of inflammation, BMS exhibited significantly lower numbers of inflammatory cells adherent to the luminal surface \((P<0.001)\) and giant cells \((P<0.001)\). Cell proliferation as indexed by anti-BrdU immunostaining showed a significantly greater percentage of positive cells in the neointima of BMS and ZES compared to EES and SES \((BMS, 3.1±1.9\%; ZES, 3.6±2.7\%; EES, 1.0±0.5\%; SES, 0.9±0.5\%; P<0.001)\) (supplemental Figure 1). Similar trends of cell proliferation also were noted in the media compared to BMS \((P=0.003)\) (supplemental Figure 1).

SEM

Re-endothelialization above struts depended on stent type, with greater coverage for ZES \((70±24\%)\), followed by EES \((66±18\%)\), and SES \((22±12\%)\) relative to BMS \((87±17\%)\; P<0.001\) (Figure 4). Stent struts lacking endothelial coverage generally showed focal aggregates of platelets and inflammatory cells, including giant cells. In contrast, areas between struts showed >88% of the luminal surface coverage, where surfaces generally were smooth with remarkably few inflammatory cells and platelets.

Expression of eNOS in BMS and Comparator DES

There was significantly decreased expression of eNOS for EES and SES relative to BMS and ZES \((BMS, 29±11\%;

Figure 2. Representative cross-sectional images of a 28-day BMS and comparator DES in the atherosclerotic rabbit iliofemoral artery. A and B, Low- (×2) and higher-power (×20) magnifications illustrating differences in strut coverage and accumulated fibrin respectively, which were both greater for EES and SES relative to ZES and BMS (low power, Movat pentachrome stain; higher power, Carstairs staining for fibrin). C, The lesion characteristics beneath the stent. Higher magnifications (insets) demonstrated greater accumulated cholesterol clefts with DES relative to BMS, which on the contrary, consisted mostly of macrophage foam cells (hematoxylin-eosin magnification, ×20). D, Calcification of the underlying plaque (arrowheads), which was greater in ZES and SES relative to BMS and EES (hematoxylin-eosin magnification, ×20). E, Immunostaining for lesional macrophages by RAM11 (magnification ×20). F, Bar graphs representing data for cholesterol clefts, calcification, and macrophages for the neointima and underlying plaque.
ZES, 27±13%; EES, 16±13%; SES, 4±5%; P<0.001) (Figure 5). No expression of eNOS was detected in segments in which the primary antibody was omitted or in smooth muscle cells (not shown), whereas nonstented control segments exhibited >60% of total surface area positivity for eNOS. At the subcellular level, eNOS protein appeared as grainy perinuclear aggregates with more of a diffuse pattern that extended into the cytoplasm. This pattern of staining was conserved independent of the stent.

**Discussion**

In the present study, biological responses of comparator 28-day DES implanted in atherosclerotic rabbit iliac arteries remarkably showed similar trends in neointimal growth and healing as reported in humans.1,2,22–25 In this regard, neointimal area was greatest for BMS and consistently less for all comparator DES where minimal thicknesses were observed for EES and SES followed by ZES, which is consistent with reported trends in recent clinical trials examining late loss at 9 months. A decrease in neointimal growth was further indicated by a reduction in cell proliferation identified by BrdU staining, which followed similar trends where the percentage of positive cells was significantly lower in EES and SES than in ZES and BMS.

A greater suppression of neointimal growth, however, was at the expense of poor stent strut coverage, which has been reported in autopsy studies to be the best predictor of late stent thrombosis.10 The number of uncovered stent struts was highest for SES followed by EES, whereas endothelial coverage was near complete for BMS. Of the 3 comparator DES devices, ZES showed the greatest amount of endothelial coverage above struts at 70%, which was nearly similar for EES and least for SES (22%). In contrast, endothelial recovery as measured by expression of eNOS was significantly greater for BMS and ZES than for EES and SES.

Therefore, our atherosclerotic rabbit data indicate that the predicted incidence of very late stent thrombosis in human would be least with ZES, which agrees with the results of a recent pivotal clinical trial of Endeavor IV at 3 years using the Academic Research Consortium definition of definite and probable stent thrombosis rate of 0.1% reported for ZES.26 Moreover, all-comer registries of ZES showed a very low (0.7%) rate of late stent thrombosis at 2 years, whereas similar registries of SES showed a much higher incidence (2.3%) at 2 years.3 Finally, in a recent optical coherence tomographic study at 9 months, ZES showed significantly fewer uncovered struts than SES (0.6±1.5% versus 16.2±17.8%, respectively; P=0.001).27 Therefore, the rabbit atherosclerotic model may be a better predictor of clinical outcome than the normal coronary artery porcine model, which is most commonly used to assess stent safety and efficacy.

**The Rabbit Atherosclerotic Model**

The rabbit model of atherosclerosis has existed since 1908 because of its unique sensitivity to hypercholesterolemia, which produces foam cell-rich lesions.28 Nevertheless, our laboratory and others have shown that lesion morphology can be modified to yield more fibrotic plaques by focal balloon injury, varying the concentration of cholesterol (0.2% to 0.25%), and extending the duration of feeding.29–31 The primary advantages of the rabbit model are a more rapid lesion development and low maintenance cost. Using the current rabbit model of atherosclerosis, we have reported biological response to oral administration of prednisone15 and pioglitazone16 following stenting, with significant reduction in neointima with both treatments compared to placebo control.
The current atherosclerotic rabbit model appears more relevant to distinguish differences between DES and BMS than a model of normal rabbits examined at 28 days, where a previous report by Nakazawa et al.\(^{32}\) essentially showed no differences in neointimal thickness among ZES, SES, and BMS at a similar time point. Studies in normal swine coronary arteries also failed to show a significant reduction in the neointimal thickness or percent stenosis between DES and BMS using a low stent-to-artery ratio of 1.1:1. However, in the pig model of greater injury (1.3 to 1.4:1), Carter et al.\(^{33}\) showed a reduction in neointimal area at 28 days, with late catch-up at 90 and 180 days. Nevertheless, differences in neointimal thickness in the present model of atherosclerotic rabbit plus balloon injury (1.3 to 1) were reminiscent of trends reported in clinical trials with less neointimal growth in all DES compared to BMS; however, delayed healing in our model was least in ZES relative to BMS and significantly greater for SES and EES.

Although neointimal suppression is accompanied by less target lesion revascularization in clinical studies, this suppression is accompanied by poor endothelialization\(^{4,8}\) as previously shown in rabbit at 14 and 28 days in single and overlapping stents.\(^{31,22}\) For the current atherosclerotic model, we specifically assessed whether re-endothelialization in response to DES was equally suppressed by SEM analysis and expression of eNOS as determined by immunohistochemistry. In our examination, endothelial coverage above stent struts was reduced in DES relative to BMS, which was least for SES compared to ZES and EES. Moreover, eNOS expression in regions between stent struts was greatest in BMS and ZES and significantly less in EES and SES. These results are consistent with clinical reports of endothelial reactivity assessed in patients with ZES and SES\(^{34,35}\) where proximal and distal segments of SES-stented arteries showed vasoconstriction in response to acetylcholine challenge or pacing. On the contrary, ZES-stented arteries exhibited vasodilatation, indicating that the adjoining endothelium (albeit outside the stent) was functional with regard to nitric oxide synthesis and release.\(^{34,35}\) In addition to the drug and dose,
Stent strut thickness is another determinant of re-endothelialization as shown by in vitro experiments from Simon et al. These researchers showed that the extent of endothelial coverage was greater with metal thicknesses of $100 \mu m$. In this regard, of the comparator DES tested, stent strut thickness of SES is greatest ($140 \mu m$) compared to ZES ($91 \mu m$) and EES ($81 \mu m$) and, therefore, is expected to have the least endothelial coverage.

The extent of macrophage infiltration within the neointima of all DES as identified by RAM11 immunostaining was significantly less relative to BMS, which consistently exhibited greater numbers of macrophages. This finding agrees with recent studies showing selective clearance of macrophages by autophagy in culture and atherosclerotic plaques of the rabbit by inhibitors of mammalian target of rapamycin, in particular everolimus, while populations of smooth muscle cells remain unaffected. Therefore, considering that ZES and EES are rapamycin derivatives with a mechanism of action similar to sirolimus (ie, mammalian target of rapamycin inhibition and degradation of p27kip1, a cyclin-dependent kinase inhibitor), the reduction of intimal macrophages was predictable. The increased apoptosis and autophagy of resident macrophages also could lead to the accumulation of cholesterol clefts, which were more frequent in the underlying plaque of DES than BMS. Moreover, it is also possible that cellular mechanisms involving cell death pathways (eg, autophagy) may result in mineralization of underlying plaque because there was significantly greater calcification in ZES and SES while absent or minimal in BMS and EES.

**Study Limitations**

Although the present model of atherosclerosis in rabbit shares several features of lesion development with human disease, such as the presence of macrophage-derived foam cells and early necrosis, these processes are not entirely representative of lesions responsible for acute coronary syndromes. Moreover, the achieved levels of circulating cholesterol levels in rabbit are considerably high, which do not accurately represent the lower values applicable to humans. The extent of underlying plaque was variable and may have resulted in

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**Figure 5.** Representative *en face* confocal images of immunofluorescent staining for eNOS from a 28-day BMS and comparator DES implanted in the atherosclerotic rabbit iliofemoral artery. A, Confocal images of eNOS showing reduced staining in comparator DES relative to BMS (green channel, eNOS; blue channel, nuclear counterstain magnification $\times 20$). B, An example of positive staining in a nonstented control segment and a negative control with omission of the primary antibody. The majority of staining in the positive control is perinuclear with varying degrees of diffuse cytoplasmic staining (magnification $\times 20$). Quantitative measurement of eNOS is shown in the table.

<table>
<thead>
<tr>
<th>Treatment (28-days)</th>
<th>BMS n=18</th>
<th>ZES n=8</th>
<th>EES n=8</th>
<th>SES n=6</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confocal Microscopy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eNOS expression, %</td>
<td>$29 \pm 11$</td>
<td>$27 \pm 13$</td>
<td>$16 \pm 13^*$</td>
<td>$4 \pm 5^*$</td>
<td>$&lt;0.001$</td>
</tr>
</tbody>
</table>

* Significantly different from BMS and ZES.
differences in neointimal healing not necessarily caused by the drugs alone. Additionally, it cannot be excluded that differences in underlying plaque area among stents represent a drug effect related to macrophage death, accumulated free cholesterol, and calcification. In the present study, stents were not examined beyond 28 days, thus precluding assessment of long-term catch-up. Similarly, endothelial regrowth is significantly greater in our model and occurs more rapidly than in humans, although these differences follow the human pattern. Finally, our studies of endothelial functionality by expression of eNOS do not precisely match functional studies in human where vasomotion generally is assessed proximal or distal to the stent, although angiography studies corroborate our findings in the atherosclerotic model.34,35

Summary and Perspective

The present findings suggest that evaluation of DES in the atherosclerotic rabbit is more predictive of neointimal progression and healing than normal rabbit iliac or porcine coronary arteries at 28 days because they more closely resemble those of late loss in randomized clinical trials. Consistent with these observations, there were greater numbers of uncovered struts in SES followed by EES and were least with ZES. Similarly, endothelial coverage was greater in ZES than EES and SES, which paralleled evidence of endothelial recovery by expression of eNOS. The results suggest greater safety for rapid-release ZES (Endeavor) than for SES (Cypher) and EES (Xience V); however, this is achieved at the expense of greater neointimal formation. Therefore, supportive experimental and clinical evidence of greater arterial healing in response to ZES may obviate the requirement for extended dual antiplatelet therapy, which needs to be confirmed in clinical trials.

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Disclosures

Drs Wilcox and Melder and Mr Pruitt are employees of Medtronic CardioVascular and own stock options in the same. Dr Virmani receives consulting fees from Medtronic CardioVascular, Abbott Vascular, Terumo Medical Corporation, and Atrium Medical Corporation. CVPath Institute has research grants from the Medtronic Vascular, Terumo Medical Corporation, and Atrium Medical Corporation. Drs Nakazawa, Nakano, Otsuka, and Kolodgie have no corporation. CVPath Institute has research grants from the Medtronic Vascular, Terumo Medical Corporation, and Atrium Medical Corporation. Dr Virmani receives consulting fees from Medtronic CardioVascular, Abbott Cardiovascular and own stock options in the same. Dr Virmani is a shareholder in a company involved in drug-eluting stent manufacturing. Drs Nakazawa, Nakano, Otsuka, and Kolodgie have no corporation. CVPath Institute has research grants from the Medtronic Vascular, Terumo Medical Corporation, and Atrium Medical Corporation. Dr Virmani receives consulting fees from Medtronic CardioVascular, Abbott Cardiovascular and own stock options in the same. Dr Virmani is a shareholder in a company involved in drug-eluting stent manufacturing. Drs Nakazawa, Nakano, Otsuka, and Kolodgie have no corporation. CVPath Institute has research grants from the Medtronic Vascular, Terumo Medical Corporation, and Atrium Medical Corporation. Dr Virmani receives consulting fees from Medtronic CardioVascular, Abbott Cardiovascular and own stock options in the same. Dr Virmani is a shareholder in a company involved in drug-eluting stent manufacturing.

References


**CLINICAL PERSPECTIVE**

Drug-eluting stents (DES) have shown success in reducing restenosis; however, late stent thrombosis has emerged as a potential cause of increased morbidity and mortality in a few patients. We compared sirolimus- (SES [Cypher]), zotarolimus- (ZES [Endeavor]), and everolimus- (EES [Xience V]) eluting stents to bare metal stent (BMS) in a rabbit model of atherosclerosis at 28 days to better predict the responses seen in humans. The extent of neointimal area and thickness was significantly less in SES and EES but in between for ZES compared to BMS. Additionally, the number of uncovered stent struts was greater in SES and EES compared to ZES and BMS. ZES was associated with the least fibrin deposition among DES. All DES significantly reduced macrophage infiltration in the neointima compared to BMS. The endothelial coverage by scanning electron microscopy was significantly greater in BMS, ZES, and EES than in SES, but endothelial nitric oxide synthase expression by confocal microscopy was greater in BMS and ZES than in EES and SES; thus, the rabbit atherosclerotic model showed greater reduction of neointimal generation in SES and EES with greater fibrin deposition, but delayed healing was much less in ZES.
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SUPPLEMENTAL MATERIAL

Supplemental Methods

Stent Placement

Under fluoroscopic guidance, stents were implanted over lesions in both iliac arteries using respective nominal pressures to achieve a visual target balloon-to-artery ratio of approximately 1.3:1. After stent implantation, post-procedural angiography was performed to document vessel patency. Anti-platelet therapy consisted of aspirin (40 mg/day) were given orally 24 h before catheterization and throughout the in-life-phase of the study, while single dose intra-arterial heparin (150 IU/kg) was administered at the time of catheterization.

Stent Harvest and Preparation

Before euthanasia, animals received bromodeoxyuridine (BrdU) to assess cellular proliferation as described previously.1 Before removal, the stented arteries were fixed in-situ at 80 mmHg with 10% neutral-buffered formalin after perfusion with lactated Ringer’s solution to remove blood. Thereafter, the stented arterial segments were harvested and further fixed by immersion (10% neutral buffered-formalin). Prior to processing, stented vessels were imaged by high-contrast film-based radiography (Faxitron X-ray Corp, Model 43855A) to assess device expansion and stent fracture.

Light Microscopy and Histomorphometry

Using the area measurements, underlying plaque and neointimal area were calculated by subtracting the stent area from EEL area, and the lumen area from stent area, respectively. The percent stenosis was calculated using the formula: stenosis % = [1- (Lumen Area/Stent Area)]*100. The number of uncovered struts was determined
based on percentage of total struts without neointimal coverage and/or surface endothelium. The extent of fibrin was semi-quantified on H&E and Carstair’s stained sections as previously described where each histology section was assigned a score of 0 to 4: 0= absence of fibrin, 1= mild, involving <10% of artery circumference or <25% of stent struts, 2= moderate, 10% to 25% of artery circumference or 25% to 50% of struts, and 4= severe, > 25% of artery circumference or >50% of struts. In addition, para-strut fibrin is also reported as a percentage of the total number of struts for each section. Percentages of para-strut giant cells were similarly reported. Advanced stages of atherosclerosis were defined by the presence of cholesterol clefts and are reported as an overall incidence for each stent type. Regional calcification was semi-quantified using a score of 0 to 4 based on an absence or number of circumferential quadrants involved.

The neointimal cell proliferation index (percent proliferating cells) in the media and neointima was defined as the ratio of BrdU-positive nuclei to total cell nuclei per high power field at (x20 magnification). Regional macrophages within the neointima and underlying plaque were identified by RAM11 immunostaining and quantified by image threshold analysis.

**Scanning Electron Microscopy**

Halved stents bisected longitudinally were rinsed in 0.1mmol/L PBS (pH 7.2) and then post-fixed in 1% osmium tetroxide for 30 minutes. Specimens were then dehydrated in a graded series of alcohol, critical point-dried, and sputter-coated with gold. The specimens were visualized using a Hitachi Model 3600N scanning electron microscope.
Immunohistochemical Detection of endothelial nitric oxide synthase (eNOS)

Halved stents bisected longitudinally were immunostained with an antibody directed against endothelial nitric oxide synthase (eNOS) (dilution 1:1000, BD Biosciences, CA) by overnight incubation at 4°C. Specific binding was visualized using a secondary antibody consisting of a donkey-anti-mouse Alexa Fluor® 488 (1:200 dilution, Invitrogen Corp, Carlsbad, CA); TOTO-3 iodide was used to counterstain nuclei. The specimens were mounted face down on glass slides, and representative images were acquired with a Zeiss Pascal confocal microscope.


**Supplemental Table 1.** *Corresponding matrix for stent allocation used for individual analysis.*

<table>
<thead>
<tr>
<th>Stent Type</th>
<th>Serum Cholesterol</th>
<th>Light Microscopy</th>
<th>SEM</th>
<th>Confocal (eNOS Expression)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMS</td>
<td>28</td>
<td>10</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>ZES</td>
<td>18</td>
<td>10</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>EES</td>
<td>18</td>
<td>10</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>SES</td>
<td>15</td>
<td>9*</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

Animals/Stents: 44/79, 20/39, 24/40†

*One artery scheduled to receive a SES implant was found totally occluded; no stent was deployed.*

† *Eight stent halves were immunostained for CD31 and are not part of this investigation.*

Abbreviations: BMS= bare metal stent, ZES= zotarolimus-eluting stent, EES= everolimus-eluting stent, SES= sirolimus-eluting stent
Supplemental Figure 1. Representative images of immunostaining for BrdU for cell proliferation from a 28-day bare metal stent (BMS) and comparator drug-eluting stents (DES) implanted in the atherosclerotic rabbit ilio-femoral artery. (Blue: nucleus, Red: BrdU positive cell)

<table>
<thead>
<tr>
<th>Treatment (28-days)</th>
<th>BMS (n=10)</th>
<th>ZES (n=10)</th>
<th>EES (n=10)</th>
<th>SES (n=9)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrdU positive cell in neointima, %</td>
<td>3.1 ± 1.9</td>
<td>3.6 ± 2.7</td>
<td>1.0 ± 0.5*</td>
<td>0.9 ± 0.5*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BrdU positive cell in media, %</td>
<td>3.8 ± 2.7</td>
<td>2.0 ± 0.7</td>
<td>1.5 ± 1.2</td>
<td>0.6 ± 0.9†</td>
<td>0.003</td>
</tr>
</tbody>
</table>

* Significantly different from BMS and ZES