JACC: CARDIOVASCULAR INTERVENTIONS VOL. 3, NO. 1, 2010

ISSN 1936-8798/10/$36.00
PUBLISHED BY ELSEVIER INC. DOI: 10.1016/j.jcin.2009.09.015

Anti-CD34 Antibodies Immobilized on the Surface of Sirolimus-Eluting Stents Enhance Stent Endothelialization

Gaku Nakazawa, MD,* Juan F. Granada, MD,† Carlos L. Alviar, MD,†
Armando Tellez, MD,† Greg L. Kaluza, MD, PHD,† Margaret Yoklavich Guilhermier, BS,‡
Sherry Parker, PHD,‡ Stephen M. Rowland, PHD,‡ Frank D. Kolodgie, PHD,*
Martin B. Leon, MD,§ Renu Virmani, MD*

Gaithersburg, Maryland; New York, New York; and Fort Lauderdale, Florida

Objectives In this study, we hypothesized that an antihuman-CD34 antibody immobilized on the surface of commercially available sirolimus-eluting stents (SES) could enhance re-endothelialization compared with SES alone.

Background Previous experience with antihuman-CD34 antibody surface modified Genous stents (GS) (OrbusNeich Medical, Fort Lauderdale, Florida) has shown enhanced stent endothelialization in vivo.

Methods In the phase 1 study, stents were deployed in 21 pig coronary arteries for single stenting (9 vessels: 3 GS, 3 SES, and 3 bare-metal stents) and overlapping stenting with various combinations (12 vessels: 4 GS+GS, 4 SES+SES, and 4 GS+SES) and harvested at 14 days for scanning electron and confocal microscopy. In phase 2, immobilized anti-CD34 antibody coating was applied on commercially available SES (SES–anti-CD34, n = 7) and compared with GS (n = 8) and SES (n = 7) and examined at 3 and 14 days by scanning electron/confocal microscopy analysis.

Results In phase 1, single stent implantation showed greatest endothelialization in GS (99%) and in bare-metal stent (99%) compared with SES (55%, p = 0.048). In overlapping stents, endothelialization at the overlapping zone was significantly greater in GS+GS (95 ± 6%) and GS+SES (79 ± 5%) compared with the SES+SES (36 ± 14%) group (p = 0.007). In phase 2, SES–anti-CD34 resulted in increased endothelialization compared with SES alone at 3 days (SES–anti-CD34 36 ± 26%; SES 7 ± 3%; and GS 76 ± 8%; p = 0.01), and 14 days (SES–anti-CD34 82 ± 8%; SES 53 ± 20%; and GS 98 ± 2%; p = 0.009).

Conclusions Immobilization of anti-CD34 antibody on SES enhances endothelialization and may potentially be an effective therapeutic alternative to improve currently available drug-eluting stents. (J Am Coll Cardiol Intv 2010;3:68–75) © 2010 by the American College of Cardiology Foundation

From the *CVPath Institute, Inc., Gaithersburg, Maryland; †Skirball Center for Cardiovascular Research, Cardiovascular Research Foundation, New York, New York; ‡OrbusNeich Medical, Fort Lauderdale, Florida; and the §Cardiovascular Research Foundation, New York, New York. This study is supported, in part, by OrbusNeich Medical. Drs. Parker and Rowland and Ms. Guilhermier are employees of OrbusNeich Medical. Dr. Virmani has received company-sponsored research support from Medtronic AVE, Abbott Vascular, Conor Medsystems, OrbusNeich Medical, Terumo Corporation, Cordis Corporation, BioSensors International, Prescient Medical, Biotronik, and Alchimedics, and is a consultant for Medtronic AVE, Abbott Vascular, Prescient Medical, and Biotronik. The first two authors contributed equally to this work.

Manuscript received May 8, 2009; revised manuscript received September 3, 2009, accepted September 20, 2009.

Re-endothelialization after vascular injury results either from local recruitment of adjacent endothelial cells (ECs) (1) or from adhesion of blood-derived endothelial progenitor cells (EPCs) that differentiate and populate the surface of the stent (2). Although the predominant mechanism of stent endothelialization is still unclear, the biological process of vascular healing after bare-metal stent implantation is known to be predictable and injury-dependent (3). In the setting of drug elution, however, the pattern of vascular

See page 76

healing is altered as there is a dynamic interaction between the prolonged inhibitory effect caused by the drug on smooth muscle and EC proliferation (4,5). Therefore, a major challenge in the development of any drug-eluting stent platform is to maintain sustained inhibition of smooth cell proliferation while promoting EC coverage.

Previous experience with stents having immobilized antihuman CD34 antibody on the device surface (Genous Bio-engineered R stent, OrbusNeich, Fort Lauderdale, Florida) has shown enhancing stent endothelialization in vitro (6) as well as feasibility and safety in the clinical setting (7,8). In this study, we tested the hypothesis that antihuman CD34 antibody immobilized on the surface of a commercially available sirolimus drug-eluting stent (SES) would enhance the degree of stent endothelialization compared with the SES surface alone. Using the healthy coronary porcine model, we evaluated the differences in surface endothelialization between bare-metal stents (BMS), anti-CD34 antibody stents (Genous stent [GS]), and SES in single and overlapping stents. Also, stent endothelialization patterns were compared between commercially available devices (SES and GS) and investigational SES with immobilized anti-CD34 antibody (SES–anti-CD34).

Methods

In vitro EC studies. One biological effect of sirolimus on EC function was evaluated by measuring the degree of proliferation of human coronary artery endothelial cells (HCAECs) and EPCs at different concentrations of the drug using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay (9). HCAECs were seeded in a 24-well plate at a density of 10 to 20,000 cells/well and grown for 72 h in the presence of different concentrations of sirolimus ranging from 0 to 1 mmol/l. Dilutions of sirolimus were prepared in endothelial growth media-2 microvascular + 20% fetal bovine serum at the following final concentrations (0 nmol/l, 1 nmol/l, 10 nmol/l, 100 nmol/l, 1 μm, 10 μm, 100 μm, 1 mmol/l); 50 μl of each dilution were added to triplicate wells of a 96-well tissue culture-treated plate and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed. Each assay was repeated at least 3 times. For the EPC proliferation study, $CD34^+$ cells were immunomagnetically separated from human peripheral blood mononuclear cells. Selected $CD34^+$ cells were then expanded for 7 days in Stemline II Hematopoietic Stem Cell Expansion Medium (Sigma-Aldrich, St. Louis, Missouri) with StemSpan CC110-Cytokine Cocktail (Stem Cell Technologies, Vancouver, British Columbia, Canada) and G-CSF (Sigma-Aldrich). Cell phenotype was further confirmed by staining with anti-CD34 antibody before use in cell studies. EPC were seeded in a 24-well plate with fibronectin-coated wells, at a density of 10 to 20,000 cells/well and were grown for 5days in the presence of 0 to 1 mmol/l of sirolimus after the same procedure used for the HCAEC incubation.

Device description. The technical features of the GS have been previously published (8). In brief, the GS consist of a 316-l stainless steel R stent (R stent) and Evolution 2 balloon delivery catheter (OrbusNeich Medical, Inc.), with a surface modification consisting of polysaccharide base matrix and covalently bound anti-CD34 antibody. The anti-CD34 antibody is an IgG2a antibody directed toward the class III epitope of the CD34 membrane protein expressed on progenitor cells. A class III anti-CD34 isoform was selected based on its cross-reactivity with porcine species, high binding affinity for CD34, and its resistance to enzymatic degradation. The amount of anti-CD34 antibody attached to the surface is 1 $\mu g/cm^2$ (1 μg/13 mm stent and 1.1 μg/15 mm stent) (Brian Hatcher, unpublished data, 2006). The distribution of the coating has been shown to be uniform when evaluated by fluorescence microscope and scanning electron microscope (SEM) with the use of a secondary fluorescein isothiocyanate- or immunogold-labeled antibody (antimurine antibody). Devices used in these studies included commercially available 2.75, 3.0, and 3.5 × 13 and 15 mm SES (Cypher, Cordis Corp., Miami Lakes, Florida), GS (Genous Bio-Engineered R Stent, OrbusNeich), and BMS (BX Sonic Stent, Cordis Corp.). The investigational SES–anti-CD34 stents were prepared by partial inflation of the sterile SES to remove them from stent delivery catheter. The removed SES were treated with the same anti-CD34 antibody immobilization process used for the GS. The modified stents were crimped on Evolution 2 stent delivery catheters (OrbusNeich) and sterilized with gamma irradiation. To

Abbreviations and Acronyms

BMS = bare-metal stent(s)
CM = confocal microscopy
EC = endothelial cell
EPC = endothelial progenitor cell
GS = Genous stent(s)
HCAEC = human coronary artery endothelial cell
PECAM = platelet endothelial cell adhesion molecule
SEM = scanning electron microscope
SES = sirolimus-eluting stent(s)
SES–anti-CD34 = sirolimus-eluting stents with immobilized anti-CD34 antibody

confirm that the drug content remained within the acceptance criteria (±10% of reported nominal) for the SES, SES–anti-CD34 stents were characterized before and after gamma sterilization by extraction in 100% methanol and high pressure liquid chromatography analysis. The total drug content was within 10% of the reported nominal drug content (8.5 μg/mm), which is comparable to the original SES. In addition, antibody activity, determined by binding of $CD34^+$ cells, of test devices was comparable to control GS devices.

Experimental design. The study protocol was reviewed and approved by the Institutional Animal Care and Research Committee, Jack H. Skirball Center for Cardiovascular Research (Orangeburg, New York). All animals received humane care in compliance with the Animal Welfare Act and the "Principles of Laboratory Animal Care" formulated by the Institute of Laboratory Animal Resources (National Research Council, NIH Publication No. 85-23, revised 1996).

PHASE 1 STUDY. Stents were randomized and deployed in normal coronary arteries in 7 pigs (21 coronary arteries) and harvested at 14 days (Fig. 1). All 7 pigs were sacrificed at day 14 and analyzed for endothelialization using SEM and confocal microscopy (CM). Of 21 vessels examined at 14 days, 9 single (3 GS, 3 SES, 3 BMS) and 12 overlapping stents (4 GS+GS, 4 GS+SES, 4 SES+SES) were deployed in the coronary arteries and analyzed by SEM and CM. For immuno-fluorescent staining followed by CM analysis, stented segments were stained for platelet endothelial cell adhesion molecule (PECAM)-1. The deployment technique involved implanting the distal stent first followed by the proximal stenting, achieving an overlapping area of ~20% to 30% of the length of the stent (i.e., ~5 mm). Therefore, the distal stent was the "predominant surface" in contact with the vessel wall in the overlapping zone.

PHASE 2 STUDY. The biological effect of the anti-CD34 antibody applied on the surface of SES was tested in a separate study (Fig. 1). In this study, a total of 22 single stents were implanted in 13 pigs. Seven pigs were sacrificed at 3 days (12 stents; 4 GS, 4 SES, 4 SES–anti-CD34) and 6 pigs at 14 days (10 stents; 4 GS, 3 SES, 3 SES–anti-CD34). All stents were assessed by SEM (visual estimation for endothelialization) and CM (PECAM-1 expression).

Stent implantation procedure. A total of 20 juvenile pigs (40 to 50 kg) were included. All animals were pre-treated with a daily dose of 325 mg aspirin and 75 mg clopidogrel for 3 days before stent implantation. Sedation was achieved with a combination of ketamine (25 mg/kg), xylazine (2 mg/kg), and acepromazine (0.2 mg) by intramuscular injection. An intravenous line was established, and the animals were intubated and ventilated with oxygen (2 l/min) and isoflurane 1.5% to 3.0% (2 l/min) using a respirator. Surgical access was obtained via femoral artery. Before catheterization, heparin (5,000 to 10,000 U) was administered to maintain an activated clotting time of 250 to 300 s. Nitroglycerin was administered intra-arterially to prevent or relieve vasospasm. A vascular introducer sheath of appropriate size was placed in the access artery for advancement of angioplasty guiding catheters. Vascular devices were implanted based on angiographic analysis of the vessel size. After allocation of the vessel to an experimental group, the appropriate stent was delivered to the intended site over a guidewire using fluoroscopic guidance, and stent deployment was performed using a 1.1:1 stent-to-artery ratio. In the overlap study, a second stent was implanted proximal to the first stent with an overlap zone of approximately 5 to 6 mm. After the procedure, the catheters were removed, the artery ligated, and the surgical incision repaired. Animals were recovered and housed until their designated day of euthanasia (3 or 14 days).

Histological analysis. Immediately after the surgical procedure on the scheduled sacrifice day, the animals were euthanized under general anesthesia by intravenous injection of pentobarbital euthanasia solution (100 mg/kg). Hearts were excised and pressure-perfused with 0.9% saline until cleared of blood, followed by pressure-perfusion fixation with 10% neutral-buffered formalin for 30 min. The hearts were fixed overnight. High-contrast film-based radiographs (Model 43855A, Faxitron X-ray Corp., Lincolnshire, Illinois) were performed to locate and assess device placement.

SEM ANALYSIS. Intact stented arterial segments were bisected longitudinally to expose the lumen surface and photographed. One-half of the stent was processed for SEM with the opposite side reserved for immunostaining and confocal imaging. Specimens were rinsed in 0.1 mmol/l sodium phosphate buffer (pH 7.2 ± 0.1) and then post-fixed in 1% osmium tetroxide for approximately 30 min. Specimens were then dehydrated in a graded series of ethanol. After critical point drying, the tissue samples were mounted and sputter-coated with gold. The specimens were visualized using a Hitachi Model 3600N SEM (Tokyo, Japan). Low power photographs of 15× were taken of the lumen surface to estimate the degree of endothelialization of the implant. Regions of interest were photographed at incremental magnifications of 50×, 200×, and 600×. Composites of serial en face SEM images acquired at low power (×15) were digitally assembled to provide a complete view of the entire luminal stent surface. The images were further enlarged (×200 magnification) allowing direct visualization of ECs. The extent of endothelial surface coverage above and between stent struts (Fig. 1, right panels) was traced and measured by morphometry software (IPLab, BD Bioscience Bioimaging, Rockville, Maryland) and reported as percent endothelial coverage. In the overlapping stent study, each stent combination underwent evaluation of the overlapping zones as well as the nonoverlapping zone. An average of each stent segment in the nonoverlapping zones

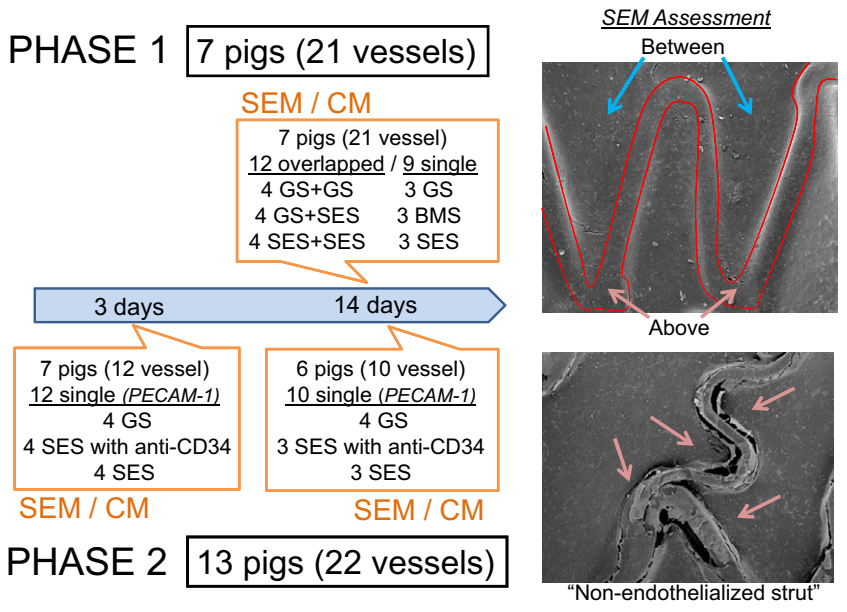

Figure 1. Study Design Diagram

Phase 1 was conducted to demonstrate endothelialization rates and endothelial functionality in single (sirolimus-eluting stents [SES], Genous stents [GS], bare-metal stents [BMS]) and overlapped SES and GS. Phase 2 was conducted to demonstrate that anti-CD34 antibody application on the surface of SES improved re-endothelialization as compared with SES alone at both 3 and 14 days. All stents were assessed by scanning electron microscope (SEM) (visual estimation for endothelialization) and confocal microscopy (CM) (platelet endothelial cell adhesion molecule [PECAM]-1 expression).

was obtained from the sum of each specific stent type (SES and GS segments).

CM ANALYSIS. CM was used to evaluate the presence of mature ECs (presence of CD31/PECAM-1 positive cells). Explanted vessels were stained for CD31/PECAM-1 (MCA 1746, Serotec, Duesseldorf, Germany). TOTO-3 (T3604, conjugated by fluoro642/660, Molecular Probes, AB Applied Biosystem, Invitrogen Corp., Carlsbad, California) was used as counter-stain (concentrations: CD31/PECAM-1 = 1:40, TOTO = 1:100). Alexa Fluor 488 donkey anti-mouse (Invitrogen Corp., A21202, Carlsbad, California) was used as a secondary antibody for CD31/PECAM-1 with concentration of 1:200. Specimens were mounted on glass slides, and representative images were acquired with a Zeiss Pascal confocal microscope. Low power images of the luminal surface were collected using a 10× objective to estimate the degree of expression of EC markers. Representative fields from the proximal, mid, and distal ends were imaged for each stent with the observer blinded to treatment. The percentage of CD31-positive

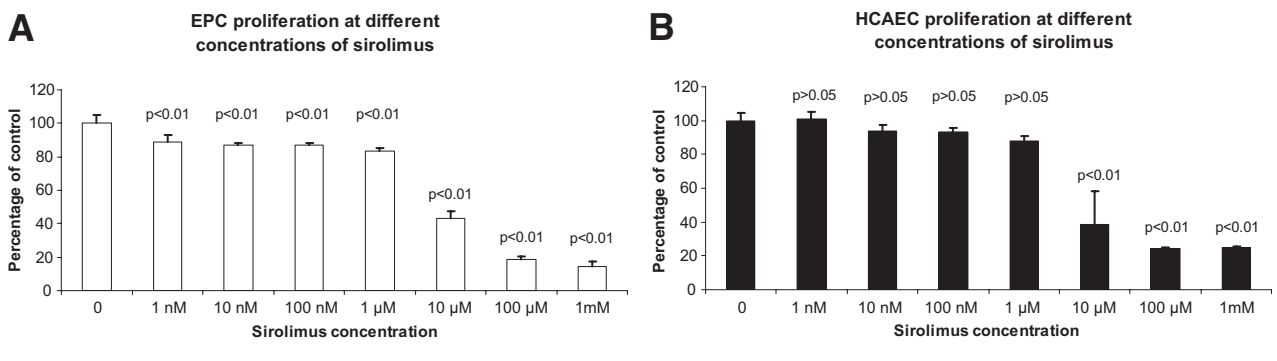

Figure 2. Effect of Sirolimus on EPC and HCAEC Proliferation at Different Concentrations

(A) Endothelial progenitor cell (EPC); and **(B)** human coronary artery endothelial cell (HCAEC). In vitro, sirolimus exerted similar cytostatic effects on both EPCs and HCAECs. However, a lower dose of sirolimus resulted in a significant decrease in proliferation of EPCs compared with control cells.

Table 1. SEM Analysis From Phase 1: Single Stenting

Stent Type	GS (n = 3)	BMS (n = 3)	SES (n = 3)	p Value
Endothelialization				
Above struts	99 ± 2	99 ± 2	55 ± 19*	0.048
Between	99 ± 1	99 ± 1	88 ± 8*	0.048

*Significantly different from Genous stent (GS) and bare-metal stent (BMS).
SEM = scanning electron microscopy; SES = sirolimus-eluting stent(s).

cells was estimated in between and above stent struts at proximal, overlapped site, and distal regions (10). Proximal and distal surface of the nonstented regions served as control regions.

Statistical analysis. For the cell studies, the means of the absorbance readings were normalized to % of control (media only), and statistical analyses included a 1-way analysis of variance and post-hoc analyses with a Dunnett's test. For calculation of half maximum inhibitory concentration values, a nonlinear regression analysis was performed, utilizing a sigmoidal dose response curve with the following equation: Y = Bottom + (Top − Bottom)/(1 + 10^[(Log IC_{50} − X)·HillSlope]); where X is the logarithm of the drug concentration, Y is the response, and ^ is exponent (GraphPad Prism 5.0, San Diego, California).

For pre-clinical studies in the porcine model, data were expressed as a mean ± SD. Statistical comparisons were performed using nonparametric test (Wilcoxon test for 2 groups and Kruskal-Wallis test for 3 or more groups). A p value of ≤0.05 was considered statistically significant.

Results

In vitro: sirolimus effect on endothelial cell proliferation. In the in vitro cell culture studies, $CD34^+$ cell (EPC) proliferation started to decrease (11.3% reduction at 1 nmol/l compared with the control group, $p < 0.05$) at lower doses of sirolimus compared with HCAEC (0% reduction at 1 nmol/l compared with the control group, p = NS). In the HCAECs, cell inhibition appeared to be affected significantly at levels above 10 μm. In both groups, there was significant inhibition of cell proliferation above 100 μm. In general, the $-\log IC_{50}$ of sirolimus was higher for $CD34^+$ cells (5.14 ± 0.03) than for HCAECs (4.80 ± 0.05), suggesting that EPC may be more sensitive to the effects of sirolimus than more mature HCAEC (Fig. 2).

In vivo studies: phases 1 and 2 studies in the porcine coronary artery model. PHASE 1: ASSESSMENT OF SINGLE AND OVERLAPPING STENTS WITH GS, SES, AND BMS. SINGLE STENTS AT 14 DAYS (SEM). A total of 9 stents (3 GS, 3 SES, and 3 BMS) were analyzed. At 14 days, stent surface endothelialization evaluated by SEM showed a significantly higher extent of endothelial coverage above struts in the GS (99 ± 2%) and the BMS (99 ± 2%) compared with the SES (55 ± 19%, p = >0.048) (Table 1).

OVERLAPPING STENT AT 14 DAYS (SEM AND CM). At 14 days, a total of 12 overlap combinations were included in the analysis of stent endothelialization (4 GS+GS, 4 GS+SES, and 4 SES+SES). SEM analysis of the overlapping zone showed an enhanced rate of endothelial coverage above the struts in the GS+GS group (95 ± 6%) and the GS+SES group (79 ± 5%) compared with the SES+SES group (36 ± 14%, p = 0.007) (Table 2, Fig. 3). The nonoverlapping segments from all 3 combinations showed higher endothelialization rates above the struts in the GS segments (98 ± 3% in GS+GS combination, 100 ± 0% in GS+SES combination) as compared with the SES segments (62 ± 33% in GS+SES combination and 46 ± 20% in SES+SES combination, p = 0.0003) (Table 2). CM analysis showed a slightly greater expression of CD31/PECAM-1 above the stent surface in the overlapping zone of GS+GS group (56 ± 13%) and GS+SES group (38 ± 33%) compared with the SES+SES group (25 ± 23%); however, the

Table 2. Endothelialization by SEM From Phase 1: Overlapping and Nonoverlapping

Overlapping Stent Zone			Nonoverlapping Stent Zone		
Stent Group (Proximal-Distal)	Location	Area Covered (%)	Stent Segment	Location	Area Covered (%)
GS–GS (n = 4)	Above struts	95 ± 6	GS segments (n = 8)	Above struts	98 ± 3
	Between	95 ± 5	(in GS–GS)	Between	99 ± 4
GS–SES (n = 4)	Above struts	79 ± 5	GS segment (n = 4)	Above struts	100 ± 0
			(in GS–SES)	Between	100 ± 0
	Between	89 ± 3	SES segment (n = 4)	Above struts	62 ± 33
			(in GS–SES)	Between	88 ± 7
SES–SES (n = 4)	Above struts	36 ± 14	SES segments (n = 8)	Above struts	46 ± 20
	Between	74 ± 10	(in SES–SES)	Between	85 ± 10
p value above struts		0.007			0.0003
p value between		0.009			0.0006

Abbreviations as in Table 1.

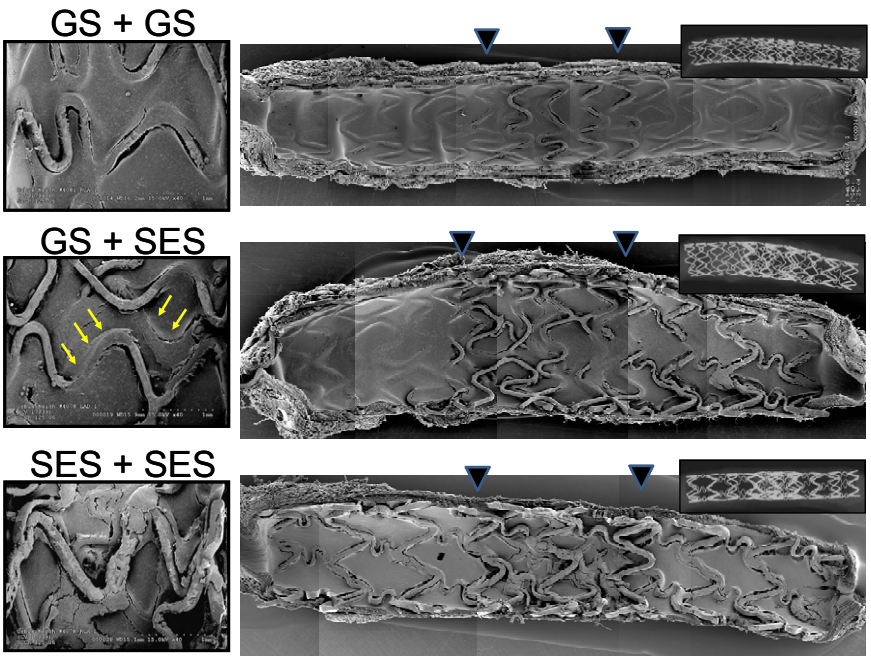

Figure 3. Representative SEM Images From Phase 1 Study Involving Overlapping Stents

Arrows point to the area of overlap and higher magnification of the overlap is shown on the left. GS+GS overlapping stents showed near complete endothelialization except for the overlapping site. Then least endothelialization is noted in the SES+SEM overlap. Abbreviations as in Figure 1.

differences did not reach a statistical significance (p = 0.27). These differences, though not significant, may have biological relevance. In the nonoverlapping zones, CD31/PECAM-1 expression was significantly greater in the GS segments (74 ± 28%) compared with the SES segments (40 ± 36%, p = 0.01).

PHASE 2: EVALUATION OF STENT ENDOTHELIALIZATION ON THE SES, GS, AND SES-ANTI-CD34. EARLY ENDOTHELIALIZATION AT 3 DAYS (SEM AND CM). A total of 12 stents (4 GS, 4 SES, and 4 SES–anti-CD34 stents) were implanted in 7 pigs and harvested at 3 days. GS showed the greatest endothelialization (76 ± 8%) among groups, and there was also an increase in endothelial strut coverage in the SES–anti-CD34 (36 ± 26%) as compared with the SES group (7 ± 3%) (p = 0.01) (Table 3, Fig. 4A). Confocal microscopic analysis of the stent surface demonstrated abundant expression of CD31/PECAM-1 over the surface of the GS group whereas the SES–anti-CD34 and the SES showed minimal expression (Table 3, Fig. 4B).

ENDOTHELIAL RECOVERY AT 14 DAYS (SEM AND CM). A total of 10 stents (3 SES–anti-CD34 stents, 4 GS, and 3 SES) were implanted in 6 pigs and harvested at 14 days. Endothelialization above stent struts was nearly completed in the GS (98 ± 2%) at 14 days, and was also increased in the SES–anti-CD34 stent (78 ± 3%) as compared with the SES group (53 ± 20% p = 0.02) (Table 3, Figs. 4A and 4C). Similarly, CD31/PECAM-1 expression was almost complete in the GS group (96 ± 7%) and also increased in the SES–anti-CD34 stent group (72 ± 8%) but remained low in the SES group (41 ± 28%, p = 0.03) (Table 3, Fig. 4B).

Table 3. SEM and CM Analysis From Phase 2: Single Stenting

Stent Type	GS	SES–Anti-CD34	SES	p Value
Endothelialization (SEM)				
3 days (n = 4 each)				
Above struts	76 ± 8	36 ± 26*	7 ± 3*†	0.01
Between	86 ± 5	78 ± 3	61 ± 9*†	0.009
14 days (n = 4/3/3)				
Above struts	98 ± 2	82 ± 8	53 ± 20*†	0.02
Between	100 ± 0	96 ± 2	94 ± 4*	0.03
PECAM-1 expression (CM)				
3 days (n = 4 each)				
Above struts	59 ± 25	1 ± 2*	0 ± 0*	0.009
Between	81 ± 14	64 ± 5*	50 ± 8*	0.009
14 days (n = 4/3/3)				
Above struts	96 ± 7	72 ± 8	41 ± 28*	0.03
Between	100 ± 0	97 ± 3	88 ± 9*	0.03

*Significantly different from GS; †significantly different from SES with immobilized anti-CD34-antibody (SES–anti-CD34).

CM = confocal microscopy; PECAM = platelet endothelial cell adhesion molecule; other abbreviations as in Table 1.

JACC: CARDIOVASCULAR INTERVENTIONS, VOL. 3, NO. 1, 2010
JANUARY 2010:68–75

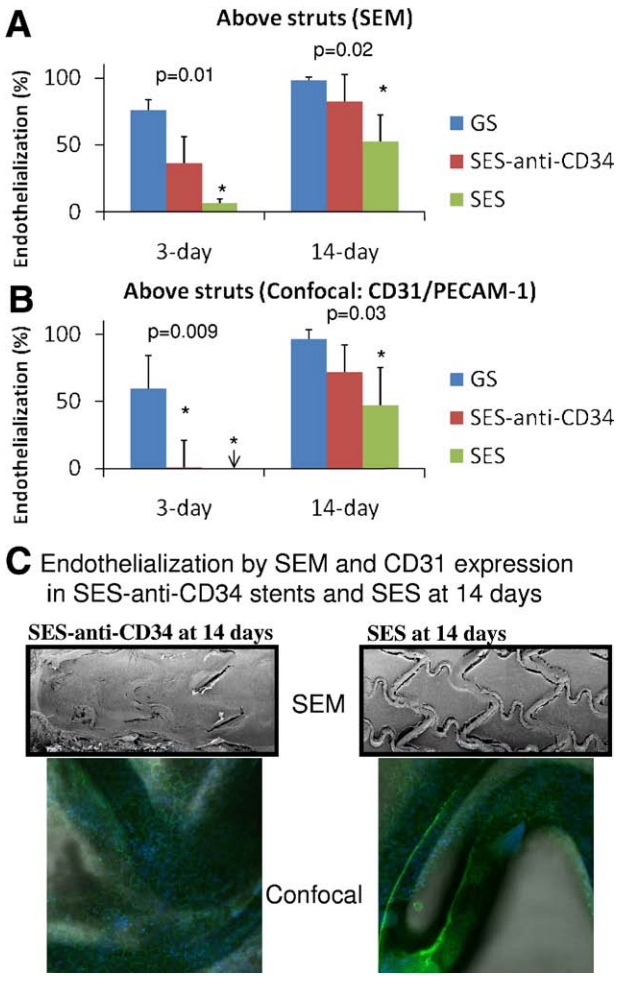

Figure 4. Endothelialization by SEM and CD-31 Expression in SES–Anti-CD34 Stents and SES at 14 Days

(A) Endothelialization by SEM. **(B)** CD31/PECAM expression by confocal microcopy harvested at 3 and 14 days. *Significantly different vs. GS. **(C)** Representative images from SEM and confocal microscopy, SES–anti-CD34 stent versus SES 14 days. Note, endothelial coverage in SES–anti-CD34 is greater than that in SES by SEM. Also a greater CD31/PECAM-1 expression **(green channel)** observed in SES–anti-CD34 stent as compared with SES alone. Abbreviations as in Figure 1.

Discussion

Re-endothelialization after stent implantation and vascular injury is a critical step in the process of vascular healing. The origin of new endothelium may be from either the adjacent recruitment of ECs (1) or from blood-derived EPCs that populate the luminal surface of the stent (2). In the setting of drug-eluting stent implantation, however, this pattern of vascular healing is altered as there is nonselective inhibition of all cell types (i.e., smooth muscle and ECs) involved in the process of stent coverage (4,5). Therefore, a paradox in the development of drug-eluting stent technologies is that the beneficial effect of drug elution is overshadowed by the nonselective inhibition of stent endothelialization (11–14). In the present study we aimed to determine if antihuman-CD34 antibodies immobilized on the surface of a commercially available SES could enhance the degree of stent endothelialization compared with the SES surface alone.

Previous experience with stents with immobilized antihuman CD34 antibody on the device surface (Genous Bio-engineered R stent, OrbusNeich) has shown enhancing stent endothelialization in vitro (6) as well as feasibility and safety in clinical setting (7,8). The in vitro cell culture study demonstrated that EPCs are more sensitive to the effects of sirolimus compared with mature human coronary artery EC. In fact, in our study, EPCs required a lower dose of sirolimus concentration to induce a similar level of cell inhibition. In the same study (data not shown), the $-\log(IC_{50})$ found for human coronary smooth muscle cell inhibition was very similar to the concentration needed to inhibit EPC proliferation to the same level (5.14 ± 0.03 for EPC, 5.23 ± 0.08 for human coronary smooth muscle cell, and 4.80 ± 0.05 for HCAEC). Therefore, the biological findings of the antiproliferative effect of sirolimus on smooth muscle cells are similar to the effects on ECs and EPCs (15–17). It is likely that by increasing EPC adhesion on the surface of a drug-eluting stent, a greater vascular healing could be achieved while maintaining therapeutic tissue levels of the drug for smooth muscle cell inhibition.

In single stents, we demonstrated that endothelialization rates were dramatically lower in the SES group compared with GS and BMS. Consistent with single stents, the overlapping SES displayed a lower rate of EC coverage especially at the midoverlap region. This finding may be of clinical relevance where overlapping stents showed significantly delayed arterial healing. Importantly, this delayed re-endothelialization in SES was ameliorated partially by overlapping with GS, thus suggesting that combination therapy of antiproliferative drug and prohealing treatments could be combined. Our findings were further reinforced by confocal microscopic analysis, which revealed an increased presence of mature ECs when assessed for PECAM-1 expression in GS+SES overlapping segments as compared with SES+SES segments. There is a disparity between % endothelialization by SEM and % PECAM-1 positive (by CM analysis), which was consistent with our previous study (10). This may be related to the fact that ECs with poor intercellular junctions display a lack of PECAM-1 expression at endothelial margins, though these areas are considered endothelialized by SEM. PECAM-1 is a transmembrane glycoprotein concentrated in broad areas of cell-to-cell contact where homophilic interactions with neighboring ECs occur (18,19). Therefore, a decrease of PECAM-1 expression suggests an impaired endothelial recovery, maturity, and functionality.

In the subsequent phase 2 study, we successfully demonstrated that anti-CD34 antibody application on the surface of SES improved re-endothelialization as compared with SES alone at both 3 and 14 days. Of note, the endothelialization rates of the GS were approximately 2-fold greater than the SES–anti-CD34 stent at 3 days. Endothelialization was almost completed by 14 days in the GS and increased by ~45% (up to 70%) in the SES–anti-CD34 stent. CM demonstrated that in the GS group most of the endothelium showed well-formed intercellular junction with CD31/PECAM-1 being expressed at 3 days in contrast to the minimal positive staining seen in the SES–anti-CD34 and in the SES groups. However, by 14 days, the presence of mature endothelium was ~80% in the SES–anti-CD34 stent and ~40% in the SES group, thus showing that although endothelialization is delayed in the SES–anti-CD34 stent at an early time point as compared with the GS, at 14 days the SES–anti-CD34 stent shows comparable endothelialization and its maturation relative to GS, which is significantly faster than the SES alone.

Conclusions

Sirolimus inhibits EPC, SMC, and mature EC proliferation. However, our study demonstrates that it is feasible to combine antiproliferative therapies with technologies designed to increase stent endothelialization, particularly EPC-capturing, with the objective of promoting vascular healing (20,21). Furthermore, anti-CD34 antibody surface modification did enhance stent endothelialization when applied on SES. In conclusion, these studies demonstrated that antibody-mediated EPC capture can enhance EC coverage and maturation of ECs on drug-eluting stents and may provide a new therapeutic approach.

Reprint requests and correspondence: Dr. Renu Virmani, CVPath Institute, Inc., 19 Firstfield Road, Gaithersburg, Maryland 20878. E-mail: rvirmani@cvpath.org.

REFERENCES

1. Robinson KA, Roubin G, King S, Siegel R, Rodgers G, Apkarian RP. Correlated microscopic observations of arterial responses to intravascular stenting. Scanning Microsc 1989;3:665–78, discussion 678–9.
2. Banerjee S, Brilakis E, Zhang S, et al. Endothelial progenitor cell mobilization after percutaneous coronary intervention. Atherosclerosis 2006;189:70–5.
3. Grewe PH, Deneke T, Machraoui A, Barmeyer J, Muller KM. Acute and chronic tissue response to coronary stent implantation: pathologic findings in human specimen. J Am Coll Cardiol 2000;35:157–63.
4. Joner M, Finn AV, Farb A, et al. Pathology of drug-eluting stents in humans: delayed healing and late thrombotic risk. J Am Coll Cardiol 2006;48:193–202.
5. Nakazawa G, Finn AV, Virmani R. Vascular pathology of drug-eluting stents. Herz 2007;32:274–80.
6. Shirota T, Yasui H, Shimokawa H, Matsuda T. Fabrication of endothelial progenitor cell (EPC)-seeded intravascular stent devices and in vitro endothelialization on hybrid vascular tissue. Biomaterials 2003;24:2295–302.
7. Co M, Tay E, Lee CH, et al. Use of endothelial progenitor cell capture stent (Genous bio-engineered R stent) during primary percutaneous coronary intervention in acute myocardial infarction: intermediate- to long-term clinical follow-up. Am Heart J 2008;155:128–32.
8. Aoki J, Serruys PW, van Beusekom H, et al. Endothelial progenitor cell capture by stents coated with antibody against CD34: the HEALING-FIM (Healthy Endothelial Accelerated Lining Inhibits Neointimal Growth-First In Man) registry. J Am Coll Cardiol 2005; 45:1574–9.
9. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
10. Joner M, Nakazawa G, Finn AV, et al. Endothelial cell recovery between comparator polymer-based drug-eluting stents. J Am Coll Cardiol 2008;52:333–42.
11. Jeremias A, Sylvia B, Bridges J, et al. Stent thrombosis after successful sirolimus-eluting stent implantation. Circulation 2004;109:1930–2.
12. Moses JW, Leon MB, Popma JJ, et al. Sirolimus-eluting stents versus standard stents in patients with stenosis in a native coronary artery. N Engl J Med 2003;349:1315–23.
13. Stone GW, Ellis SG, Cox DA, et al. One-year clinical results with the slow-release, polymer-based, paclitaxel-eluting TAXUS stent: the TAXUS-IV trial. Circulation 2004;109:1942–7.
14. Tsimikas S. Drug-eluting stents and late adverse clinical outcomes lessons learned, lessons awaited. J Am Coll Cardiol 2006;47:2112–5.
15. Butzal M, Loges S, Schweizer M, et al. Rapamycin inhibits proliferation and differentiation of human endothelial progenitor cells in vitro. Exp Cell Res 2004;300:65–71.
16. Chen TG, Chen JZ, Wang XX. Effects of rapamycin on number activity and eNOS of endothelial progenitor cells from peripheral blood. Cell Prolif 2006;39:117–25.
17. Zhao FH, Chen YD, Jin ZN, Lu SZ. Are impaired endothelial progenitor cells involved in the processes of late in-stent thrombosis and re-endothelialization of drug-eluting stents? Med Hypotheses 2008;70:512–4.
18. Piali L, Hammel P, Uherek C, et al. CD31/PECAM-1 is a ligand for alpha v beta 3 integrin involved in adhesion of leukocytes to endothelium. J Cell Biol 1995;130:451–60.
19. Finn AV, Joner M, Nakazawa G, et al. Pathological correlates of late drug-eluting stent thrombosis: strut coverage as a marker of endothelialization. Circulation 2007;115:2435–41.
20. Finn AV, Nakazawa G, Joner M, et al. Vascular responses to drug eluting stents: importance of delayed healing. Arterioscler Thromb Vasc Biol 2007;27:1500–10.
21. Sandhu G, Doyle B, Singh R, et al. Frequency, etiology, treatment, and outcomes of drug-eluting stent thrombosis during one year of follow-up. Am J Cardiol 2007;99:465–9.

Key Words: stent ■ vascular healing ■ anti-CD34 antibody ■ sirolimus ■ EPC.