Impact of Stent Surface on Thrombogenicity and Vascular Healing
A Comparative Analysis of Metallic and Polymeric Surfaces

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Background—Emerging drug-eluting stent technologies are evolving toward the elimination of polymeric component used as the method for modulating drug delivery. Although this technological approach seems to be biologically appealing, the impact of durable polymers and metallic stent surfaces on vascular healing remains unclear. In the present study, we aimed to compare the independent effect of a durable polymer and a metallic stent surface on thrombogenicity and endothelial cell coverage using different in vitro and in vivo experimental models.

Methods and Results—Platinum chromium (PtCr) and polyvinylidene fluoride-co-hexafluoropropene (PVDF-HFP)–coated surfaces were evaluated in this study. Thrombogenicity was assessed by exposing all surfaces to human blood under shear flow conditions. The inflammatory potential of the material was evaluated by measuring cytokine release from THP-1 cells exposed to all surfaces for 24 hours. Endothelial cell coverage was evaluated by detection of CD31 after the stents were exposed to human coronary artery endothelial cells for ≤14 days. Platelet adhesion (P<0.01) and activation (P=0.03) on PVDF-HFP were greater than on PtCr. In vivo, PVDF-HFP revealed more neointimal area (P<0.01) and residual parastrut fibrin (P=0.01) at 30 days compared with PtCr. PtCr displayed higher endothelialization rates and higher vascular endothelial-cadherin expression at 7 and 14 days (P=0.02) compared with PVDF-HFP.

Conclusions—Thrombogenicity and vascular healing differ among metallic and polymeric stent surfaces. PVDF-HFP exhibits higher degrees of platelet activation–adhesion and thrombus accumulation in vivo compared with PtCr. PtCr displayed higher degrees of endothelial surface coverage compared with PVDF-HFP surfaces. (Circ Cardiovasc Interv. 2013;6:370-377.)

Key Words: endothelial cells • drug-eluting stents • polymers • thrombosis • vascular diseases

Drug-eluting stents (DES) have dramatically reduced the long-term rate of reintervention and improved clinical outcomes among patients undergoing percutaneous coronary interventions.1 In large randomized controlled trials, second-generation DES further improved clinical outcomes but still carried a low but important risk of late thrombotic events.2 Autopsy and in vivo human imaging studies suggest delayed healing as the most important mechanism of late stent thrombosis.3 At the present time, the independent contribution of each different DES component (metal versus polymer) to the process of vascular healing is still unclear.

New-generation DES technologies have evolved into thinner metallic scaffolds covered with lower amounts of bioabsorbable polymers as drug carriers. Although the concept of converting a DES into a bare-metal stent is theoretically appealing, there are few data comparing the biological effect of different stent surfaces on vascular healing. In the present study, we aimed to compare the independent effect of the stent surface on platelet behavior, thrombogenicity, and healing response using different in vitro and in vivo experimental models.

Methods

Test Articles and Polymer Coating

Test Articles
Flat disks and strips were fabricated from platinum chromium (PtCr; Carpenter Technology, Reading PA) and were cut from sheets by electric discharge machining. Final dimensions of disks were 1.70 cm (0.680 inches) in diameter, and final dimensions of strips were 8.89 cm (3.5 inches)×0.782 cm (0.308 inches)×0.079 cm (0.031 inches).

Received January 2, 2013; accepted May 23, 2013.

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The online-only Data Supplement is available at http://circinterventions.ahajournals.org/lookup/suppl/doi:10.1161/CIRCINTERVENTIONS.113.000120/-/DC1.

Circ Cardiovasc Interv is available at http://circinterventions.ahajournals.org

DOI: 10.1161/CIRCINTERVENTIONS.113.000120
WHAT IS KNOWN

• Drug-eluting stents containing nonresorbable polymers have decreased restenosis rates but remain associated with delayed healing and very late stent thrombosis.

• Drug-eluting stents containing resorbable polymers decrease restenosis rates in a manner comparable with drug-eluting stents with nonresorbable polymers; however, the impact of resorbable polymers on thrombogenicity and healing has not been established fully.

WHAT THE STUDY ADDS

• Metallic stent surfaces are less thrombogenic than polyvinylidene fluoride-co-hexafluoropropene-only–coated surfaces.

• Polyvinylidene fluoride-co-hexafluoropropene-only–coated surfaces are associated with delayed stent endothelialization compared with metallic stent surfaces.

• The preferential deposition (aboluminal) of bioresorbable polymers may decrease stent thrombogenicity and enhance vascular healing after drug-eluting stent implantation.

Polymer Coating of Test Articles
Thin polyvinylidene fluoride-co-hexafluoropropene (PVDF-HFP) polymer films were applied to strips, disks, and stents (3.0 mm×16 mm ELEMENT PtCr stent; Boston Scientific, Minneapolis, MN) using a spray coater (Boston Scientific Ireland Ltd and Ballybrit Industrial Estate, Co, Galway, Ireland). Test articles were held in fixtures, and the spray coater applied the PVDF-HFP coating per the PROMUS (Boston Scientific, Minneapolis, MN) production settings to apply thin conformal films. The surface areas of the strips and disks were measured and scaled to a 16-mm stent to apply the same total coating weight per unit surface area, producing a total polymer thickness of ∼4 to 6 μm. Subsequently, all PVDF-HFP–coated test articles were dried to remove the residual solvents in the coatings and sterilized by ethylene oxide.

Platelet Surface Staining and Image Analysis
Platelet activation and adhesion to the flat disks were evaluated by exposing freshly drawn human whole blood to physiological arterial shear stress. Disks were housed in a custom assembly and placed into a commercially available cone and plate-shearing device (Diamed Impact-R; Biorad Diamed GmbH, Switzerland). Human blood was collected and characterized as described in detail in the online-only Data Supplement. Platelet adhesion and activation on PtCr and PVDF-HFP–coated disks were evaluated with blood from each donor (n=6 donors). Blood (130 μL) was added to each disk, and shear stress (1800 s⁻¹ shear rate for 5 minutes) was applied according to the manufacturer’s specifications. As a positive control, gold-coated disks were also tested.45 Disks were washed with 1× PBS (Life Technologies, Carlsbad, CA), placed in 4% paraformaldehyde (Sigma Aldrich, St. Louis, MO) for 30 minutes, followed by incubation in 10% goat serum (Life Technologies, Carlsbad, CA) to block nonspecific binding of antibodies. Adherent cells were then treated with unlabeled mouse anti-human primary antibodies to detect CD41a (Exalpha Biologicals, Maynard, MA), CD62P, and fluorescence images (4 per disk, with 90° rotation of disk between image fields) of 850 nm×650 μm were obtained 4 μm from the edge of each disk to minimize edge effects. Images were captured using an Olympus BX61WI microscope. Percent platelet surface coverage in each image was quantified with Metamorph Image Analysis Software (Molecular Devices, Sunnyvale, CA) and used to represent platelet adhesion (CD41a) and activation (CD62P).

Evaluation of Inflammatory Potential
PtCr and PVDF-HFP–coated flat strips were treated with human platelet-poor plasma for 1 hour at room temperature, removed, and placed in a manifold creating 8 separate wells (0.32 cm² surface area) equally spaced along the length of each strip. Incubation of test articles with plasma was performed to better represent the in vivo condition where proteins are absorbed from plasma onto the material surface after exposure to blood. As a positive control, gold-coated strips were included, because gold induces leukocyte activation and inflammation.57 Undifferentiated human monocytic cells (THP-1; American Tissue Type Culture, MD) were placed in each well of each strip (200 μL/0.32 cm² surface area) at 10⁶ cells/mL in serum-free RPMI-1640 (American Type Culture Collection, Manassas, VA) and incubated under standard cell culture conditions for 24 hours. Next, cell viability was assessed by trypan blue exclusion, and cell-conditioned medium from each well was analyzed for interleukin-8, interleukin-1β, and tumor necrosis factor-α abundance using commercially available fluorescent bead-based immunoassay multiplex kit according to manufacturer’s specifications (Milliplex Map, St. Charles, MO). Interleukin-1β, tumor necrosis factor-α, and interleukin-8 are potent proinflammatory cytokines that may have a negative impact on vascular healing.8–10 Cell viability was >90% and did not differ among the materials tested. Fluorescence was measured with a Luminex 100 (Luminex Corporation, Austin, TX) and correlated to cytokine concentrations using a standard curve generated with a 5-parametric logistic curve fitting program (IS 2.3, Luminex Corporation). The cytokine levels among the 8 wells per strip (1 strip per test group per experiment) were averaged.

Blood Loop Assessment of Stent Thrombogenicity
The assessment of thrombus formation, including platelet, fibrin, and total cellular accumulation, was determined in uncoated and PVDF-HFP–coated PtCr stents (3.0 mm×16 mm, identical ELEMENT stent platform). A closed blood loop consisting of silicone tubing (Cole Parmer) connected to a pulsatile blood pump (Harvard Apparatus, Holliston, MA) was placed in an incubator at 37°C. Stents and tubing were incubated overnight in platelet-poor plasma at 37°C before testing. Fresh human blood was collected as previously described, and it was placed on a rocking platform at room temperature. Immediately before testing stents, blood was treated with calcium chloride (Sigma) and magnesium chloride (Sigma) at concentrations of 3.4 and 2.7 mmol/L, respectively. Activated clotting time was determined using a HemoChron Response (International Technidyne Corporation, Edison, NJ), and it ranged from 140 to 190 seconds for blood used in these experiments.

Cellular Responses to Stent Materials

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PtCr test articles were processed using the commercial production procedures for ELEMENT stents. All test articles were sterilized by ethylene oxide before analyses. X-ray photoelectron spectroscopy was performed on flat test articles to evaluate surface composition, and high-resolution scans were performed to analyze major alloy elements using a PHI Quantera XPS system (Physical Electronics, Chanhassen, MN). X-ray photoelectron spectroscopy was also performed on representative stents, demonstrating comparable surface composition with flat test articles (data not shown). As positive controls for platelet and inflammatory cell responses, 316L stainless steel strips and disks of identical dimensions to the test articles with plasma was performed to better represent the in vivo condition where proteins are absorbed from plasma onto the material surface after exposure to blood. As a positive control, gold-coated strips were included, because gold induces leukocyte activation and inflammation.57 Undifferentiated human monocytic cells (THP-1; American Tissue Type Culture, MD) were placed in each well of each strip (200 μL/0.32 cm² surface area) at 10⁶ cells/mL in serum-free RPMI-1640 (American Type Culture Collection, Manassas, VA) and incubated under standard cell culture conditions for 24 hours. Next, cell viability was assessed by trypan blue exclusion, and cell-conditioned medium from each well was analyzed for interleukin-8, interleukin-1β, and tumor necrosis factor-α abundance using commercially available fluorescent bead-based immunoassay multiplex kit according to manufacturer’s specifications (Milliplex Map, St. Charles, MO). Interleukin-1β, tumor necrosis factor-α, and interleukin-8 are potent proinflammatory cytokines that may have a negative impact on vascular healing.8–10 Cell viability was >90% and did not differ among the materials tested. Fluorescence was measured with a Luminex 100 (Luminex Corporation, Austin, TX) and correlated to cytokine concentrations using a standard curve generated with a 5-parametric logistic curve fitting program (IS 2.3, Luminex Corporation). The cytokine levels among the 8 wells per strip (1 strip per test group per experiment) were averaged.

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Blood loops containing 2 stents of the same test group arranged in parallel sections of tubing were assembled and evaluated for each donor (n=8). Stents were expanded in tubing at a stent-to-tubing-diameter ratio of ≈1.1/1.0. All stents were visualized before and after testing for apposition to the tubing wall. Tubing was filled with blood at 37°C and connected to the pump. Blood was perfused through each stent at a flow rate of 100 mL/min for 5 minutes. Thereafter, the blood was removed, and each section of tubing with the stent was perfused with Tyrode buffer and photographed. Sections of tubing containing stents were cut and placed in 1% Triton-X to lyse cells. Aliquots of lysed cells were assayed for CD31 (marker for platelet accumulation) and lactate dehydrogenase (LDH; marker of total cell accumulation) using commercial assays according to manufacturer’s recommendations (CD31 ELISA; Abnova, Taipei City and LDH; Promega, Madison, WI). For fibrin measurements, stents were exposed to plasmin (0.5 U/mL, Sigma) for 6 hours at 37°C, and the level of D-dimer was determined by immunoassay (ASSERACHROM D-Di; Diagnostica Stago, Parsippany, NJ). Values of CD31, LDH, and D-dimer were average of the 2 stents within a loop.

**Endothelial Cell Coverage on Stents**

Endothelial cell (EC) growth on uncoated and PVDF-HFP–coated PtCr stents (3.0 mm×16 mm, ELEMENT) was evaluated using human coronary artery ECs (HCAECs; Cell Applications Inc, San Diego, CA), as previously described, and is described in detail in the online-only Data Supplement.

**In Vivo Comparison of Thrombotic Response and Neointima Formation**

Histological sections from porcine coronary arteries, including PtCr and PVDF-HFP–coated stents, were quantitatively analyzed and are described in detail in the online-only Data Supplement.

**Statistical Analysis**

For platelet adhesion and activation on flat test articles, differences in paired measurements were normally distributed with equal variances, as determined by the Shapiro–Wilk and Levene methods, respectively. Statistical significance was determined by ANOVA with repeated measures with multiple comparisons to PtCr using the Holm–Sidak post hoc test. Data for D-dimer of PtCr and PVDF-HFP were normally distributed with equal variance and analyzed by an unpaired t test. Absorbance values for endothelialization of PtCr and PVDF-HFP were normally distributed. Differences in absorbances for endothelialization between PtCr and PVDF-HFP at 7 and 14 days were analyzed by paired t test and Wilcoxon signed-rank test, respectively. For inflammation, endothelialization, platelet accumulation (flow loop), and LDH data, mean values for each test article group were normalized to the respective mean value for uncoated PtCr group for each experiment and were normally distributed. These data are shown as the mean with 95% confidence interval of the ratio of each test group to uncoated PtCr and are significant if the confidence interval does not include a value of 1. For the in vivo data, statistical significance was determined by Welch t test. A P<0.05 was considered to be statistically significant.

**Results**

**Platelet Adhesion, Activation, and Cytokine Release**

The disks exposed to human blood showed different patterns of platelet adhesion and activation. PtCr, PVDF-HFP–coated, and gold disks were tested simultaneously in experiments (n=6), each using blood from a single donor. A significant increase in platelet adhesion was observed on PVDF-HFP compared with PtCr (P<0.01; Figure 1A). Gold elicited an increase in platelet adhesion compared with PtCr (P<0.01).

**Figure 1.** Platelet adhesion to and activation on platinum chromium (PtCr)– and polyvinylidene fluoride-co-hexafluoropropene (PVDF-HFP)-coated surfaces. A, Platelet adhesion was elevated on PVDF-HFP and gold surfaces compared with that on PtCr (P<0.01). B, Platelet activation (n=6) on PVDF-HFP (P=0.03) and gold (P=0.05) was greater compared with that on PtCr platelet adhesion, but activation on PVDF-HFP was lower compared with that on gold. Microscopic images of fluorescently stained platelets on (C) PtCr and (D) PVDF-HFP surfaces show adhesion of platelets to PVDF-HFP surfaces. *Significantly different from PtCr (P<0.05). Values shown are mean±SEM and n=6 per group.
Platelet activation followed a similar trend, with PtCr exhibiting 2- to 3-fold less, compared with that observed on PVDF-HFP (P=0.03; Figure 1B). Gold elicited greater degrees of platelet activation compared with PtCr (P=0.05).

In addition, compared with PtCr, PVDF-HFP-coated surfaces did not induce significant changes in cytokine levels (P=0.17; Figure 2A–2C). As a positive control, gold-coated surfaces increased the level of each cytokine 2- to 3-fold compared with PtCr (P=0.03).

In Vitro Thrombus Accumulation
In the flow loop model and after the perfusion of human blood, PVDF-HFP–coated stents accumulated 50% more platelets compared with uncoated PtCr stents (P=0.04; Figure 3A). Furthermore, both D-dimer (P=0.01) and LDH (P=0.01) within the thrombus were significantly greater in PVDF-HFP–coated stents compared with uncoated PtCr stents (Figure 3B and 3C) and correlated with the gross visual observations of thrombosis between test groups (Figure 3D).

EC Coverage
A significant reduction in HCAEC coverage was observed on PVDF-HFP–coated PtCr stents compared with uncoated stents (Figure 4A and 4B). In Figure 4A, absorbance between PtCr and PVDF-HFP at 7 (P<0.01) and 14 days (median values: PtCr=0.31 and PVDF-HFP=0.24; P=0.02) was significantly different. Indeed, by 7 and 14 days, endothelialization on PVDF-HFP–coated stents was ≈40% and ≈30% lower, respectively, compared with uncoated PtCr stents (P=0.01; Figure 4B). For experiments (n=7) where stents were implanted concurrently for assessment at 7 and 14 days, the increase in endothelialization was 33±6 SEM % and 78±15 SEM % relative to 7 days for PtCr and PVDF-HFP–coated stents, respectively (P=0.02, determined by unpaired t test). The lesser change in endothelialization between 7 and 14 days for PtCr compared with PVDF-HFP reflects that the PtCr stents are likely approaching optimal EC coverage, which is supported by images of ECs on stents in Figure 5. By 14 days, endothelialization of uncoated PtCr stents seemed, by staining with 4',6-diamidino-2-phenylindole, to cover the struts completely, whereas PVDF-HFP–coated struts showed regions that were not covered (Figure 5A, 5D, and 5G). Immunohistochemical staining of HCAECs on stents at 14 days (Figure 5A–5I) showed that HCAECs covering uncoated stents demonstrated a more robust staining of vascular endothelial-cadherin at intracellular junctions (Figure 5B) compared with HCAECs on PVDF-HFP–coated stents, suggesting a reduced barrier function of HCAECs on PVDF-HFP (Figure 5E). Background staining associated with vascular endothelial-cadherin was nominal, as evidence of weak to absence of detection of the isotype control (Figure 5I). Furthermore, 4',6-diamidino-2-phenylindole staining revealed a relatively greater number of cells of ECs on uncoated stents, compared with PVDF-HFP–coated stents, and confirmed the quantitative assessment of EC coverage using CD31 expression (Figure 4A and 4B).

In Vivo Thrombotic Response and Neointima Formation
Thrombus area 7 days postimplantation and both residual thrombus and neointimal area at 30 days postimplantation, comparing PVDF-HFP polymer-only–coated PtCr-stented coronary arteries with uncoated PtCr stents, are shown in the Table. Comparing all histological sections (proximal, overlap, and distal), thrombus area at 7 days was 40% to 50% greater with PVDF-HFP–coated struts than bare PtCr struts (Table). Representative microscopic views of thrombus at 7 days comparing PVDF-HFP covered with bare PtCr stents are shown in Figure 6. At 30 days postimplantation, thrombus had been mostly organized into neointima, with residual thrombus consisting predominantly of fibrin. Representative microscopic views of fibrin and neointima at 30 days comparing PVDF-HFP–covered PtCr stents with bare PtCr stents are shown in Figure 6. Values presented in
the Table show that PVDF-HFP–covered struts are associated with 40% to 50% more neointima at 30 days compared with PtCr.

**Discussion**

DES have dramatically reduced the long-term rate of re-intervention and improved clinical outcomes among patients undergoing percutaneous coronary interventions. Although second-generation DES demonstrate improved clinical outcomes, long-term stent thrombotic events still occur. Several
strategies have been suggested to continue the further development of DES technologies. New metallic alloys (ie, PtCr) have allowed further miniaturization of the stent struts, leading to enhanced deliverability and lower degrees of vascular injury and restenosis, while maintaining radiopacity. Additional technological advances have focused on the development of DES platforms covered with small amounts of bioresorbable polymers (or no polymer) as drug carriers, modulating drug delivery while the polymeric reabsorption process occurs. However, although the concept of converting a DES into a bare-metal stent is theoretically appealing, there are little data available comparing the independent impact of different stent surfaces found in commercially available DES on thrombogenicity and vascular healing.

The importance of this study is that it compared different ex vivo and in vivo cellular response to stent biomaterials currently used in clinically available DES platforms using assays that are relevant to biological events associated with implantation of coronary stents. Specifically, the biological differences seen between PtCr (ELEMENT stent) and PVDF-HFP–coated stents (durable polymer used in the XIENCE V (Abbott Vascular) and PROMUS (Boston Scientific) everolimus-eluting stent were compared. It is generally recognized that all stent materials investigated in this study induce some degree of platelet reactivity. However, platelet adhesion and activation were observed to be significantly greater on PVDF-HFP compared with PtCr surfaces (Figure 1A–1D). The greater reactivity of blood on PVDF-HFP surfaces compared with PtCr surfaces was further supported by the blood flow loop results demonstrating greater cellular and fibrin accumulation in PVDF-HFP–coated stents (Figure 3A–3D). Both this study and that of Chin-Quee et al are in contrast to findings reported by Kolandaivelu et al, who suggested that DES polymers are less thrombogenic than their bare-metal stent counterparts. A major difference is that polymer-only–coated stents were used in this study and by Chin-Quee et al compared with drug-eluting polymer–coated stents as a representative response to polymer by Kolandaivelu et al.

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Mean neointimal areas for both stents are also presented at 30 d. Stents were deployed in an overlap configuration, and data are presented for both the overlap and nonoverlap sections. All data are mean ±SD in mm². PtCr indicates platinum chromium; and PVDF-HFP, polyvinylidene fluoride-co-hexafluoropropene.

*There were 19 sections instead of 21 sections at 7 d and 22 instead of 24 sections at 30 d because in 1 vessel in each of the PtCr groups the stents were completely overlapped so that no nonoverlapping sections could be obtained. PVDF-HFP group at 30 d includes 1 stent with severe hypersensitivity inflammatory response. The P value removing this stent was 0.032 for neointimal area and 0.028 for fibrin area at the overlap sections. For overlap and nonoverlap sections combined, the P value was 0.015 for neointimal area and 0.023 for fibrin area.
It is biologically plausible that the fully functional DES used in the Kolandaivelu study resulted in drug concentrations within the flowing blood sufficient to impair different thrombotic pathways. Indeed, it has been demonstrated that mTOR inhibitors reduce clot retraction and platelet aggregation. Furthermore, Kolandaivelu et al. used a surrogate indicator of clot mass, LDH, which is present in all blood cells and plasma. A limitation to this method is that LDH may not accurately reflect the size of a thrombus, which can be composed of different cell types (platelets, leukocytes, and erythrocytes) that contain various amounts of LDH. Indeed, erythrocytes contain >2-fold more LDH per cell than platelets. Chin-Quee et al. showed that LDH levels did not correlate with thrombus weight in stents, but were likely attributed to thrombus composition. In this study, our findings demonstrated that the increase in thrombotic accumulation on PVDF-HFP surfaces compared with PtCr is caused by increased recruitment of platelets, fibrin, and other blood derived cells.

Most importantly, the ex vivo findings were confirmed by the in vivo study in which stents containing the same stent surfaces (PtCr and PVDF-HFP) were implanted in porcine coronary arteries and examined by the presence of thrombus and neointimal thickening. The greater levels of thrombus deposits around the struts at 7 days and residual thrombus (predominantly fibrin) at 30 days (Figure 6) seen on the PVDF-HFP surfaces are consistent with increased platelet adhesion and activation observed in vitro (Figure 1). Thus, PVDF-HFP-coated struts exposed to blood flow offer a more thrombogenic surface compared with a bare luminal PtCr stent, resulting in more initial thrombus and subsequently more neointima from thrombus organization.

The significance of platelet adhesion and activation extends beyond blood coagulation to play a role in inflammation and healing. Platelet adhesion to a surface, whether an injured artery or stent material, forms a substrate onto which inflammatory cells can immobilize and migrate into surrounding arterial tissue. Subsequent liberation of chemotactic and mitogenic factors from adherent platelets and inflammatory cells can promote arterial smooth muscle cell migration and proliferation, both of which are significant contributors to restenosis. This is evident by the greater neointima formed at 30 days with PVDF-HFP-coated stent struts than with bare PtCr struts in our analysis of porcine coronary arteries (Table).

Endothelialization of uncoated PtCr was more rapid than PVDF-HFP-coated PtCr stents (Figure 4A and 4B). Barrier function of HCAECs on PtCr was more robust than cells on PVDF-HFP-coated PtCr, because the intensity of vascular endothelial-cadherin localized between HCAECs was brighter when cells covered bare PtCr compared with PVDF-HFP-coated PtCr (Figure 5B and 5E). This is an important finding because the biocompatibility of stent materials extends beyond the traditional parameters of inflammation and blood reactivity to include assessments of strut endothelialization, an important feature for the prevention of stent thrombosis. Experimental and autopsy data suggest that delayed healing, leading to uncovered struts, occurs more frequently in DES and correlates with late thrombotic events. In addition, the combinations of polymers and antiproliferative drugs used on DES have both been implicated as factors contributing to poor endothelialization. Antirestenotic agents on DES nonselectively inhibit migration and proliferation of arterial smooth muscle cells and ECs. However, few studies, such as this, and that of Chin-Quee et al. have addressed the impact of polymer on endothelialization. Assessments of stent materials to support endothelialization is limited, because current in vivo models lack the sensitivity to detect differences, and in vitro assays that directly measure adhesion and proliferation do not adequately mimic the process of strut endothelialization.

This study used an in vitro model to mimic endothelialization of stent materials that are situated adjacent to the vascular wall in vivo by evaluating endothelialization of materials embedded into a collagen matrix covered by HCAECs. In contrast, PVDF-HFP-coated stents endothelialized less significantly (Figure 4A and 4B) and with reduced expression of vascular endothelial-cadherin (Figure 5B and 5E), compared with uncoated PtCr stents. The relative lack of endothelialization is likely not because of an inability of HCAECs to populate PVDF-HFP by adherence and proliferation. These phenomena were demonstrated in a previous study in which PVDF-HFP supported EC adhesion and growth when cells were directly placed onto them. An alternative explanation for relative endothelialization deficiencies on PVDF-HFP may be that PtCr has been demonstrated to absorb significantly greater amounts of fibronecin, a chemotactic stimulus of ECs that enhances their migration, when each of these materials was exposed to human plasma.

The present study has several limitations that deserve to be described. First, there is no standardized methodology for the study of cell behavior after exposure to novel biomaterials. Second, although experimental animal models have been shown to be useful for the validation of safety of stents, their use for the prediction of efficacy and stent thrombotic events has been limited. Considering that the vascular response to injury varies between humans and porcine models, caution must be taken when interpreting these results. However, because of the fact that biomaterials currently used in clinical practice were used in all the experiments, the transition from the in vitro to the in vivo setting provides further understanding into the mechanisms of material biocompatibility and thrombosis. However, the clinical significance of these findings needs to be validated in prospective randomized human clinical studies.

In summary, the present study suggests that different stent surfaces elicit different cellular responses that may influence the overall thrombogenicity and vascular healing profile of the stent. Specifically, PtCr blood-contacting surfaces seem to be more biocompatible compared with PVDF-HFP surfaces. These findings carry important clinical implications. Although bare metal stent metals are exposed directly to tissue, durable polymers used in current DES technologies isolate the metal from the arterial tissue. Currently approved DES in the United States use circumferential durable polymeric coatings that remain in vascular tissue after drug elution is complete. Autopsy studies suggest that the permanent presence of these polymers may elicit chronic arterial inflammation, resulting in delayed healing and late thrombotic events. Although it has been shown that second-generation polymers (ie, PVDF-HFP) used in current DES provide a more biocompatible surface than early-generation
polymers and improved clinical outcomes compared with first-generation DES, important biocompatibility differences still remain compared with bare metallic surfaces. Therefore, our data suggest that short-term drug elution while polymer absorption occurs is biologically preferable to maintaining a continuous and permanent polymeric surface once drug elution has occurred. This approach offers the benefits of minimizing polymeric load, while avoiding chronic inflammatory reactions but maintaining the beneficial antiproliferative effect.

**Sources of Funding**
Boston Scientific Corporation, Natick, MA, supported this study.

**Disclosures**
Drs Eppihimer, Sushkova, Grimsby, Forhyth, Wang, Huibregtse, and Dawkins and Larsen and Efimova are employees and own stock in Boston Scientific Corporation. Dr Wilson is a paid consultant for Boston Scientific Corporation. Dr Granada has received research grants from Boston Scientific Corporation. The other authors report no conflict.

**References**
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Circ Cardiovasc Interv. 2013;6:370-377; originally published online July 30, 2013; doi: 10.1161/CIRCINTERVENTIONS.113.000120

Circulation: Cardiovascular Interventions is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1941-7640. Online ISSN: 1941-7632

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Platelet Poor Plasma Processing

Fresh human whole blood (Research Blood Components, Brighton, MA) was collected in acid citrate dextrose (15%). Blood was dispensed into conical tubes and centrifuged for 20 minutes at 2400 x g. Next, plasma was removed, placed into fresh tubes, and centrifuged again for 20 minutes at 2400 x g to obtain platelet poor plasma (PPP). Platelet-poor plasma from 4 healthy donors was pooled, aliquoted and stored at -80°C until needed for experimentation.

Blood Collection and Characterization

Human blood was drawn into 3.2% sodium citrate tubes (Becton Dickinson; Franklin Lakes, NJ) using an 18-gauge needle collection kit (Becton Dickinson, Franklin Lakes, NJ). The tourniquet was released immediately following venipuncture and the first 2-3 ml of blood drawn discarded. Platelet activation in blood samples was determined by 2-color flow cytometry (Becton Dickinson, Franklin Lakes, NJ) under both non-stimulated and ADP-stimulated conditions prior to testing on materials. Platelets were identified by positive staining for CD41a (PerCP-Cy5.5-labeled mouse anti-human CD41a, clone: HIP8, BD Biosciences, San Jose, CA). Fluorescein-isothiocyanate (FITC)-labeled antibodies (Ab) directed against CD62P (mouse anti-human CD62, clone: AK-4, BD Biosciences, San Jose, CA), activated αIibβ3 (mouse anti-human αIibβ3 clone: PAC-1, BD Biosciences, San Jose, CA) and fibrinogen (rabbit anti-human fibrinogen, clone F0111, DAKO Corporation) were used to determine platelet activation. The percentage of non-stimulated platelets demonstrating expression of CD62P, activated αIibβ3 or fibrinogen binding was on average 2-3% and did not differ from the corresponding isotype controls. Platelets were activated with ADP (20 µM; BioDATA Corporation, Horham, PA) for 2 min at room temperature, which elevated the percentage of platelets expressing CD62P, activated αIibβ3 and binding to fibrinogen to 24%, 35% and 22%, respectively. Blood was not used if platelets exhibited elevated baseline activation
above 10% in any marker and did not demonstrate a minimal 4-fold increase in each marker following ADP stimulation.

**Endothelial Cell Coverage on Stents**

Endothelial cell (EC) growth on uncoated and on PVDF-coated PtCr stents (3.0 mm x 16 mm, ELEMENT™) was evaluated using human coronary artery endothelial cells (HCAECs) (Cell Applications Inc., San Diego, CA). Stents were incubated in PPP for 1 hour at room temperature, removed, and subsequently embedded at one stent/well into the HCAEC-covered collagen gels. Stents were removed from the gels after 7 and 14 days, placed in 10% formalin, washed with PBS, and HCAECs were detected by immunostaining of CD31, an EC biomarker. For CD31 staining, HCAECs were treated with a mouse anti-human CD31 antibody (0.25 µg/ml; GTI, Brookfield, WI) or its isotype control followed by incubation with a biotin-conjugated secondary antibody (0.08 µg/ml; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and streptavidin-alkaline phosphatase conjugate (1:1; 0.2 µg/ml; Life Technologies, Carlsbad, CA). Stents were washed and exposed to a p-nitrophenyl phosphate substrate solution (Life Technologies, Carlsbad, CA). Substrate development was stopped by addition of sodium hydroxide (1N; Sigma Aldrich, St. Louis, MO). Five stents per test group per experiment were evaluated with 3 stents being stained for CD31 and 2 stents being stained with an isotype control to assess background staining. Aliquots of the substrate solution for each stent were placed into a 96-well tissue culture plate and absorption was measured in a plate reader (Molecular Devices, Sunnyvale, CA, US). Average background absorbances were subtracted from each CD31-stained stent to achieve adjusted absorption levels and average per group. Signal intensities correlated with the HCAEC coverage as observed by fluorescent microscopy.

As assessment of barrier function of HCAECs that covered stents, cell-covered stents were fixed at 14 days, as described above, and HCAECs were stained with 4',6-diamidino-2-phenylindole (DAPI, Life Technologies, Carlsbad, CA) to detect cell nuclei and localize the HCAECs, and an antibody directed against VE-cadherin, a cellular junction protein. In brief, stents were removed from the gel, fixed in 10%
formalin, washed in PBS and stained with VE-cadherin antibody (BD Biosciences, San Jose, CA) or isotype control at 10 µg/ml, followed by application of an Alexa Fluor 647 goat anti-mouse secondary antibody (10 µg/ml; Life Technologies, Carlsbad, CA). Thereafter, stents were washed and stained with 4',6-diamidino-2-phenylindole (DAPI, 300nM in PBS). Images of HCAEC on uncoated and PVDF-coated stents were collected using a confocal microscope (Olympus FlouView, Olympus, and Center Valley, PA) under equivalent instrument settings and merged with transmitted images of the underlying stent.

**In Vivo Comparison of Thrombotic Response and Neointima Formation**

Histological sections from a stented porcine coronary artery safety study (11) including bare PtCr stents (ELEMENT™ PtCr stent) and PVDF-HFP coated stents (ELEMENT™ PtCr stent) were quantitatively analyzed for the presence for para-strut thrombus, residual thrombus (predominantly fibrin) and neointimal areas out to 30 days to delineate the cellular response differences between PtCr and PVDF-HFP surfaces. In brief, uninjured porcine coronary arteries were implanted with partial overlapping stent pairs of bare PtCr and PVDF-HFP-coated stents. Histological sections (3 total from each stented artery) from the proximal and distal non-overlapping regions and the overlapping region were obtained from stented arteries at 7 days (bare PtCr, n=7, PVDF-HFP, n=8) and 30 days (bare PtCr, n=8, PVDF-HFP, n=8).

Areas of para-strut thrombus at 7 days and residual para-strut thrombus (predominantly fibrin) and neointima at 30 days were measured by tracing the regions seen on elastic-trichrome stained in-stent sections. High resolution images of the para-strut regions were obtained from sections and collaged to produce a composite image of each in-stent section with the features of interest traced using Photoshop software with image analysis “plug ins” was used to calculate areas per section.