Original Article

Effect of a Novel Stent on Re-Endothelialization, Platelet Adhesion, and Neointimal Formation

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Aim: Vascular endothelial-cadherin (VE-cadherin) is specifically expressed by outgrowth endothelial cells (OECs). Zwitterionic stent showed high antifouling and excellent blood compatibility. Therefore, we hypothesized that anti-VE-cadherin antibody-coated zwitterionic stents (VE-cad-Z stents) would promote re-endothelialization, reduce neointimal formation, and resist thrombus.

Methods: VE-cad-Z stents were examined using platelet adhesion test, platelet activation, and OEC capture ability *in vitro*. *In vivo* effect of VE-cad-Z stents on re-endothelialization, thrombus-resistance, and neointima hyperplasia was investigated in left common carotid arteries of rabbits ($n=15$).

Results: *In vitro*, VE-cad-Z stents showed better platelet-resistance and OEC-capture ability (DNA concentration: 297.23 ± 22.71 versus 67.49 ± 15.26 ng/ μ L, $P<0.01$). *In vivo*, VE-cad-Z stents exhibited better patency rate than bare metal stents (BMS) (15/15 versus 12/15), and it significantly reduced platelet adhesion and neointima formation (neointima area: 1.13 ± 0.05 versus 1.00 ± 0.05 mm², $P<0.01$ and 3.04 ± 0.11 versus 1.05 ± 0.06 mm², $P<0.01$, at 3 and 30 days, respectively; % stenosis: 20.99 ± 0.98 versus 18.72 ± 0.97 , $P<0.01$ and 56.46 ± 2.20 versus 19.45 ± 1.24 , $P<0.01$, at 3 and 30 days, respectively).

Conclusion: These data suggested that VE-cad-Z stents could specifically capture OECs, consequently promote endothelial healing, and also reduce platelet adhesion and neointima formation.

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Key words: Small diameter stent, Vascular endothelial-cadherin, Zwitterionic structures, Restenosis, Re-endothelialization

Introduction

Drug eluting stents (DES) have greatly reduced in-stent restenosis (ISR) rate of small diameter disease. However, these anti-healing stents interfere with the process of natural vascular healing by preventing or delaying the formation of a functional endothelial

monolayer over the stents and thus cause some serious safety problems like stent thrombosis and neointimal proliferation¹⁻³).

Previous studies suggested that rapid re-endothelialization of the stented area could reduce the risk of inflammation and subsequent thrombus formation⁴). Therefore, the endothelial progenitor cells (EPCs) capture stents (GenousTM Bio-Engineered R Stent, OrbisNeich, Fort Lauderdale, Florida) coated with anti-human CD34 monoclonal antibody have been developed and shown to enhance endothelialization⁵⁻⁷). However, CD34-positive cells are capable of differentiating into different kinds of cells including inflammatory cells and vascular smooth muscle cells

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(SMCs)⁸⁻¹⁰). This problem, the lack of specificity to capture EPC, may explain the increased ISR of GenousTM Bio-Engineered R Stent^{11, 12}). Therefore, cell-specific molecular targeting EPC-capture without activation of platelets or recruitment of inflammatory cells and vascular SMC is important for developing more efficient and safer DES in the future.

Recently, J.M. Lee *et al.* carried out a head-to-head comparison between anti-CD34 antibody-coated stents and anti-VE-cadherin antibody-coated stents. The results revealed that anti-VE-cadherin antibody-coated stent could capture circulating outgrowth endothelial cells (OECs) and endothelial cells more effectively and specifically *in vitro* and *in vivo*¹³). Moreover, VE-cadherin is specifically expressed in adherent junctions of endothelial cells and performs important functions such as intracellular signaling and cell–cell adhesion¹⁴). Therefore, we hypothesized that VE-cadherin may be an ideal target surface marker for capturing EPC.

Our previous study showed that zwitterionic stent had high antifouling and excellent blood compatibility using tests such as platelet adhesion tests, hemolysis assay, coagulation time tests¹⁵). Therefore, in the present study, we applied anti-VE-cadherin antibody to our newly developed zwitterionic stents. We hypothesized that these VE-cad-Z stents may enhance re-endothelialization and resist thrombus effectively.

Materials and Methods

Fabrication of VE-cad-Z Stents

Bare metal stents (BMS) were purchased from Yinyi Co. (Dalian, China), and all chemical reagents and solvents without specific note were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO). BMS-CuBr stents with atom transfer radical polymerization (ATRP) functionalities were prepared as previously described¹⁶). For the preparation of polyethylene glycol methacrylate (PEGMA) brushes on the BMS-Br surface, PEGMA (8 ml, 24.6 mmol), CuBr (0.0360 g, 0.246 mmol), CuBr₂ (0.0120 g, 0.0500 mmol), bpy (0.0760 g, 0.492 mmol) in 8 ml of DMF was added in a three-neck flask. After three cycles of filling and deflating gas, the reaction proceeded with a protection of argon at 35°C for 4 h. The resulting surface was rinsed using copious amounts of DMF, ethanol, and deionized water, followed by drying under reduced pressure. For the production of BMS-poly(ethylene glycol)-2-ethacryloxyethyl phosphorylcholine (BMS-PEG-MPC) stents, the reaction was carried out using CuBr (0.057 g, 0.390 mmol), bpy (0.125 g, 0.809 mmol), MPC (2.36 g, 7.99 mmol) in 7.5 ml metha-

nol under an argon atmosphere at room temperature for 24 h. BMS-PEG-MPC was washed thoroughly using ethanol and deionized water to ensure complete removal of the physically adsorbed reactants, before drying in a vacuum desiccator under reduced pressure overnight. Finally, 10 µg of mouse monoclonal anti-human VE-cadherin antibody (ab155350, Abcam) was used in antibody adsorption after BMS-PEG-MPC in 50 ml MES buffer (0.05 mol/l, pH 5.60) reacted with 100 mg EDC and 60 mg NHS for 10 min.

Platelet Adhesion Test *in vitro*

The BMS and VE-cad-Z stents were placed into cell culture plates. To equilibrate BMS and VE-cad-Z stents, 1 mL of phosphate buffered solution (PBS) was added in each well and allowed to remain for 12 h. After removing the PBS, 1 mL of freshly prepared platelet rich plasma (PRP) of human blood, which was prepared from citrated fresh human blood, was dropped on each well and allowed to remain for 90 min at 37°C. The PRP was removed using an aspirator, and each well was rinsed three times with 1 mL of PBS. Then, 1 mL of 2.5 vol % glutaraldehyde in PBS was added in each well, and the materials were maintained at room temperature for 30 min to fix the platelets on the stents. The stents were washed with PBS again and then subsequently dehydrated by systemic immersion in a series of ethanol–water solutions [50, 60, 70, 80, 90, 95, and 100% (v/v)] for 30 min each and allowed to evaporate at room temperature. The surfaces of stents with platelets were observed using a scanning electron microscope (SEM, Model S-2260N; Hitachi Co. Ltd.). For SEM, six identical specimens were observed, and the presented images are representative.

Platelet Activation *in vitro*

To measure the platelet activation, the PRP was incubated at 37°C with four types of samples. The incubation mixture was removed at 30 min to assess the activation state of the platelets by flow cytometry. Expression of the fluorescently labeled platelet activation marker anti-CD62P and platelet pan-marker anti-CD42a was detected using a BD FACSCalibur (BD Biosciences, USA). All the platelet activation experiments were conducted in triplicate.

Preparation of OECs

OECs were prepared as previously described¹⁷). In brief, human peripheral blood mononuclear cells (PBMCs) were re-suspended in culture medium after isolation. The PBMCs (1×10^7 /well) were then immediately plated on collagen type I (BD Biosciences)-

Fig. 1. Comparison of platelet adhesion on BMS and VE-cad-Z stents.

A, Representative SEM image of BMS after contact with PRP for 90 min. B, Representative SEM image of VE-cad-Z stents after contact with PRP for 90 min. Inserts show high-power SEM images of the stent surface. White arrow in the image denotes platelets. Bar = 10 μ m.

coated 35-mm diameter tissue culture plates (BD Biosciences). Medium was changed daily for 7 days and then every other day till the first passage. To further confirm the OECs phenotype, the cultured cells were incubated with monoclonal antibodies against CD31-PE, CD144-PE, CD34-PE (BD Bioscience), CD14-FITC, CD45-FITC (Immunotech), and CD133-PE (R&D Systems) and analyzed using a fluorescence-activated cell sorting (FACS) flow cytometer and WinMDI software (Becton Dickinson).

Evaluation of OECs Capture *in vitro*

The dynamic culture was followed as previously reported¹⁸. In brief, 10 BMS and 10 VE-cad-Z stents were connected to a circulatory loop system, which consisted of a roller pump (Zhisun Instrument Co., Ltd.) upstream of the graft and an outflow reservoir downstream of the graft. The stent was tied to the circulatory loop, and the circulatory loop was filled with culture medium (2×10^6 OECs/ml). Medium flowed from the roller pump through the stent to the outflow reservoir. Medium in the outflow reservoir was pumped up using the roller pump and recirculated. To simulate physiological flow rates, the pump setting was 70 strokes/min, which yielded a flow rate of 60 mL/min. At this flow rate, the calculated shear stress at the graft was 30 dyn/cm². The entire system was installed in an incubator at 37°C in a humidified environment with 5% CO₂. After 24 h of perfusion, the stents were retrieved for SEM observation.

DNA quantification was used to estimate the OEC-capture ability of BMS and DES. The QIAmp AllPrep DNA mini kit (Qiagen, Milan, Italy) was used for DNA preparation. DNA quantity and quality was

analyzed by reading the whole absorption spectrum (220–750 nm) with NanoDrop ND-2000 and calculating DNA concentration and absorbance ratio at both 260/280 and 230/260 nm¹⁹. To further confirm the viability of the captured OECs, they were labeled with calcein AM and DAPI staining. For this purpose, stents were washed with $1 \times$ PBS, pH 7.4 (Invitrogen), followed by staining with calcein AM (2 μ M) (Invitrogen) for 45 min at room temperature. After the removal of free calcein by washing with Hank's balanced salt solution (HBSS), the staining facilitated observation of live cells. Thereafter, cells were fixed with 4% PBS-buffered paraformaldehyde (15 min), washed twice in HBSS, and stained with 406-diamino-2-phenylindole (DAPI, 0.05 mg/ml HBSS, Sigma) for 15 min to observe the nuclear morphology.

Surgical Implantation *in vivo*

All care and handling of the animals were provided according to the Guide for the care and use of Laboratory Animals approved by the Ethical Committee of Researches of Nanjing University.

In brief, 30 male rabbits (New Zealand white) with a body weight between 3.5 and 4.0 kg were randomly implanted with BMS ($n=15$) and VE-cad-Z stents ($n=15$) in the left carotid arteries and were followed for 3 and 30 days. Anesthesia was induced with an injection of intramuscular ketamine (30 mg/kg) and intravenous pentobarbital (30 mg/kg) and then maintained using isoflurane and oxygen. Through a longitudinal left neck incision, left common carotid artery exposed. Before arterial clamping, heparin (100 U/kg) was administered intravenously. Then, a BMS or VE-cad-Z stent was implanted in the left common

carotid artery. Both stents had a nominal diameter of 2.5 mm and a length of 20 mm. The closure was sutured by layers. All animals were administered 40 mg aspirin in combination with 75 mg clopidogrel daily per os postoperatively. The implanted stent patency was monitored using a handle Doppler probe (HP Sonos 4500; Philips) every 3 days, and an angiograph was performed before they were harvested.

Morphologic Study After Implantation

Three days after implantation, five BMS and five VE-cad-Z stents were explanted for histological examination, and the remaining stents were explanted after 30 days of implantation. Midportion segments of the stents were fixed with 10% (vol/vol) buffered formaldehyde solution and dehydrated with a series of ethanol. The samples were embedded in resin and cut using a tungsten blade. The degree of neointimal growth was analyzed in the sections stained with hemotoxylin and eosin. The neointimal area (NA) and the area within the internal elastic lamina (IEL) were measured by computerized morphometry, which was carried out by a single observer who was blinded to the experimental protocol. All images were captured by an Olympus XI 70 microscope equipped with an Olympus Magna Fire digital camera and were analyzed using a computerized digital image-analysis system (Image-Pro Plus version 6.0, MediaCybernetics, Silver Spring, Maryland).

SEM studies were conducted using tissues processed as previously described²⁰. After harvest, the stents were dissected longitudinally to evaluate the luminal surface as enface, fixed in 2.5% glutaraldehyde, and processed for scanning electron microscopy for evaluation of re-endothelialization and platelet resistance. Samples were incubated with HRP-conjugated Polyclonal anti-von Willebrand factor (vWF) antibody (DakoCytomation), followed by visualization with diaminobenzidine (Sigma) to characterize the endothelium of the stents.

Western Blot Analysis

Vessel wall expression of proteins such as endothelial nitric oxide synthase (eNOS), α -smooth muscle actin (α -SM Actin), and monocyte chemoattractant protein-1 (MCP-1) was determined using western blot analysis. In brief, the stent struts were carefully removed from the coronary arteries after harvest. Then, protein extracts (50 μ g) were size fractionated on SDS-polyacrylamide gels and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). Positive expression was analyzed using antibodies against eNOS (abcam, ab95254), α -SM Actin (abcam, ab7817), and

Fig. 2. Characterization of the adhered platelets on VE-cad-Z stents and BMS.

A, The representative figure of FACS data to assess the proportion of the activated platelet on the negative control. B, The representative figure of FACS data to assess the proportion of the activated platelet on BMS. C, The representative figure of FACS data to assess the proportion of the activated platelet on VE-cad-Z stents. The expression of the platelet activation marker anti-CD62p-FITC on platelets was defined as % of platelet activation.

Fig. 3. Comparison of OECs capture ability between VE-cad-Z stents and BMS.

A-B, Representative SEM image of BMS (Fig. 3A) and VE-cad-Z stents (Fig. 3B) 24 h after dynamic culture with OECs. C, DNA quantification 24 h after dynamic culture with OECs. For statistical analysis, unpaired *t*-test was performed.

MCP-1 (abcam, ab18678). Horseradish peroxidase-conjugated donkey anti-rabbit antibody (Santa Cruz Biotechnology) or rabbit anti-mouse antibody (Santa Cruz Biotechnology) were used as the secondary antibody at a dilution of 1:2000. Antibody binding was detected on X-ray films using an enhanced chemiluminescence method. β -actin on the same membranes was used as a constitutive marker. Quantity One software (Bio-Rad) was used to determine the results of the densities.

Statistical Analysis

Data were analyzed using SPSS 21.0 (IBM Corp., Armonk, USA). Quantitative data were expressed as

mean $\pm$ SD. Statistical analysis of differences between two groups was performed using unpaired *t*-test, and the statistical analysis of differences among three or more groups was assessed using ANOVA and multiple comparison tests. A *p*-value of less than 0.05 was considered statistically significant.

Results

Platelet Adhesion Test *in vitro*

To assess preliminary blood compatibility, the platelet adhesion on BMS and VE-cad-Z stents was evaluated by SEM observation. The surfaces of BMS were porous. After contact with PRP for 90 min, the

Fig. 4. Identification of OECs and viability assessment 24 h after dynamic culture.

A-B, Representative DAPI staining image of VE-cad-Z stents (Fig. 4A) and BMS (Fig. 4B). C-D, Representative calcein-AM staining image of VE-cad-Z stents (Fig. 4C) and BMS (Fig. 4D).

surfaces of BMS were covered with platelets. The adhered platelets were highly activated with the characteristics of spreading, aggregation, and pseudopodia (**Fig. 1A**). However, the surfaces of VE-cad-Z stents were smooth, and the platelet adhesion degree decreased substantially once the zwitterionic polymer brushes were grafted onto BMS. Almost no platelets were adhered on the stent (**Fig. 1B**).

Platelet Activation *in vitro*

Activated platelets were characterized by plasma membrane display of markers such as CD62P as well as by aggregation state. We measured the platelet activation after incubating the samples in PRP for 30 min at 37°C using Flow cytometry. The BMS caused significant platelets activation; platelet activation was 13.7% higher than the negative control (1.73%) (**Fig. 2A and B**). After surface modification, approximately 0.05% of the platelets were activated (**Fig. 2C**).

OECs Capture *in vitro*

OECs capture ability of VE-cad-Z stents was examined by SEM, DNA quantification, and immunofluorescent analysis. After 24 h dynamic culture with OECs, few OECs attached on the BMS and aligned themselves disorderly (**Fig. 3A**). The cells on VE-cad-Z stents spread out completely and possessed a sustainable growth capability. OECs aligned well-organized and adhered tightly to each other to maintain the high integrity of their confluent monolayer structure, which was similar to that of the native artery (**Fig. 3B**). DNA quantification demonstrated that VE-cad-Z stents captured a significantly increased number of OECs than BMS (DNA concentration: 297.23 ± 22.71 versus 67.49 ± 15.26 ng/ μ L, $P < 0.01$, $n = 10$, **Fig. 3C**), which was consistent with the SEM results. In addition, immunofluorescent analysis confirmed that, in comparison with BMS, VE-cad-Z stents captured more viable OECs as evidenced by amplified calcein AM and DAPI fluorescent staining intensity (**Fig. 4**).

Fig. 5. A, Surgical view of stents during implantation. B, Surgical view of stents after 30 days of implantation. C, Duplex Doppler ultrasound examination of patent VE-cad-Z 30 days after implantation. D, A typical angiogram of VE-cad-Z stents 30 days after implantation. White arrow in the image denotes stented sites.

Stent Patency

Fifteen VE-cad-Z stents and 15 BMS were implanted into the carotid arteries of the rabbit. Three BMS were observed with thrombotic occlusion within 3 days by arterial Doppler ultrasound and carotid arterial angiographies, whereas all the remaining stents maintained patency till they were harvested for histological and morphometric analysis. VE-cad-Z stents exhibited excellent patency rate *in vivo* (**Fig. 5C and D**).

Platelet Resistance and Regeneration *in vivo*

SEM was conducted to study *in vivo* effects of

VE-cad-Z stents on platelet resistance and regeneration. SEM results showed the following: (1) three days after implantation, only a few OECs attached on the luminal surface of the VE-cad-Z stents, which showed almost no difference between the BMS and VE-cad-Z stents group. Regarding the platelet adhesion, there was no platelet adhered to the VE-cad-Z stents luminal surface, whereas large number of platelets were observed on the BMS luminal surface (**Fig. 6A and B**). (2) Thirty days after implantation, the luminal surfaces of the VE-cad-Z stents were covered with a confluent layer of cobblestone-like cells orienting in a par-

Fig. 6. Representative SEM images of the stents retrieved after implantation.

A-B, VE-cad-Z stents (Fig. 6A) and BMS 3 days after implantation (Fig. 6B). C-D, VE-cad-Z stents (Fig. 6C) and BMS (Fig. 6D) 30 days after implantation. White arrow in the image denotes thrombus.

allel manner to the direction of arterial flow without platelet adhesion. In contrast, an incomplete OECs layer and many platelets covered BMS luminal surface (Fig. 6C and D). Positive staining revealed vWF of the cells overlying the intimal layer, which was properly denoted as ECs, and the results of immunofluorescent analysis complied with SEM results (Fig. 7).

Neointimal Hyperplasia

Histological and morphometric analysis was performed to access the neointimal hyperplasia. Histological analysis demonstrated the following: (1) compared with VE-cad-Z stents, a significant in-stent neointima was formed at BMS stent sites 3 days after implantation (Fig. 8A and B) (2) compared with 3 days after implantation, the neointima area of BMS increased significantly 30 days after implantation, whereas VE-

cad-Z stents showed almost the same level (Fig. 8C and D). Quantitative analysis demonstrated a significant reduction in neointima formation and stenosis at the stent site of the VE-cad-Z stents (neointima area: 1.13 ± 0.05 versus 1.00 ± 0.05 mm² and 3.04 ± 0.11 versus 1.05 ± 0.06 mm² at 3 and 30 days, respectively; % stenosis: 20.99 ± 0.98 versus 18.72 ± 0.97 and 56.46 ± 2.20 versus 19.45 ± 1.24 at 3 and 30 days, respectively, Fig. 8E and F). Neointima formation and stenosis significantly increased 30 days after implantation compared with 3 days after implantation (neointima area: 1.13 ± 0.05 versus 3.04 ± 0.11 mm², % stenosis: 20.99 ± 0.98 versus 56.46 ± 2.20 , Fig. 8E and F).

Protein Expression of the Stent

Densitometric analysis of Western blotting demonstrated that the expression of α -SM Actin and

Fig. 7. Immunofluorescent staining of the stents retrieved after implantation.

A-B, Representative images of von Willebrand factor (vWF)-positive cells (green) on VE-cad-Z stents (Fig. 7A) and BMS (Fig. 7B) 3 days after implantation. C-D, Representative images of von Willebrand factor (vWF)-positive cells (green) on VE-cad-Z stents (Fig. 7C) and BMS (Fig. 7D) 30 days after implantation. Bar=0.5 mm.

MCP-1 in VE-cad-Z stents was significantly lower compared with BMS (α -SM Actin: 0.51 ± 0.09 versus 1.50 ± 0.15 and MCP-1: 0.14 ± 0.05 versus 2.30 ± 0.23 , **Fig. 9B**). α -SM Actin in VE-cad-Z stents showed almost the same level compared with the native carotid artery (NCA), but MPC-1 was still significantly higher than that in NCA (α -SM Actin: 0.51 ± 0.09 versus 0.47 ± 0.06 and MCP-1: 0.14 ± 0.05 versus 0.06 ± 0.03 , **Fig. 9B**). eNOS protein expressed in the VE-cad-Z stents was significantly higher than that in the BMS (0.49 ± 0.08 versus 0.09 ± 0.02 , **Fig. 9B**) and showed almost the same level compared with NCA (0.49 ± 0.08 versus 0.50 ± 0.06 , **Fig. 9B**).

Discussion

OECs are considered to be a good candidate for vascular regenerating cell therapy, as they have much

higher proliferative potential than early EPCs and marked similarity to completely differentiated ECs with regard to cellular morphology, marker expression, and the potential to form capillary-like structures²¹). VE-cadherin is a surface marker, which is exclusively expressed on the OECs and endothelial cells, but not on the early EPC and other leukocytes²²). In our previous reports, flow cytometry analysis demonstrated that 95.8% OECs expressed VE-cadherin on their surfaces¹⁷). Therefore, VE-cadherin may be a more ideal target surface marker for capturing EPC than CD34^{13, 23}). Biocompatible materials, containing zwitterion and PEG, have been identified as two important synthetic nonthrombogenic materials for DES^{24, 25}). PEG has received much attention because of its properties of inertness for protein adsorption and cell adhesion, steric stabilization effect, and high chain mobility and low interfacial free energy²⁶). Zwitter-

Fig. 8. Histopathological analysis of in-stent neointima formation after implantation.

A-B, Representative hematoxylin and eosin (H&E)-stained sections of VE-cad-Z stents (Fig. 8A) and BMS (Fig. 8B) 3 days after implantation. C-D, Representative hematoxylin and eosin (H&E)-stained sections of VE-cad-Z stents (Fig. 8C) and BMS (Fig. 8D) 30 days after implantation. E, The neointima area at BMS and VE-cad-Z stents. For statistical analysis, unpaired *t*-test was performed. F, The % stenosis ($100 \times \text{area of neointima} / \text{area of internal elastic lamia}$) at BMS and VE-cad-Z stents. For statistical analysis, unpaired *t*-test was performed. NS = not significant.

ionic structures are designed to mimic phosphatidylcholine, which is abundant in cell membranes. These structures contain phosphorylcholine, carboxybetaine,

and sulfobetaine chemistries and are effective in resisting thrombosis^{27, 28}). Therefore, we developed a new stent using anti-human VE-cadherin antibody to coat

zwitterionic stents.

In the present study, we demonstrated the following: (1) zwitterionic polymers resisted the platelet adherence and prevented platelet activation effectively *in vitro*, (2) VE-cad-Z stents could capture OECs significantly *in vitro*, (3) anti-VE-cadherin antibody did not affect the viability or proliferation of OECs *in vitro*, and (4) compared with BMS, VE-cad-Z stents reduced neointimal hyperplasia and thrombus significantly 3 and 30 days after stent implantation in the rabbit model.

The thrombotic risk for DES extends far beyond that for BMS²⁹⁾, and hypersensitivity reaction to polymers on DES causes activation of thrombotic cascade resulting in stent thrombosis³⁰⁾. To overcome this detrimental side effect, we designed PEG-MPC zwitterionic polymers on a molecular level and anchored PEG-MPC onto the exterior surface of BMS by ATRP functionalities. *In vitro*, we demonstrated that the platelets adhered on VE-cad-Z stents were significantly less than that on BMS and the platelet activation of VE-cad-Z stents was markedly lower than that of BMS. We hypothesized that the seaweed-like structure on the surface of BMS possessed the excellent ability to resist protein and platelet adhesion, and PEG-MPC zwitterionic polymers could maintain the natural structure and state of the plasma protein contact it, which may enhance platelet resistance of VE-cad-Z stents.

In vitro, VE-cad-Z stents also showed excellent OEC-capture ability and biocompatibility. As a result, VE-cad-Z stent struts were covered with a confluent monolayer structure that was similar to the native artery after 24h dynamic culture with OECs. The DNA concentration of the VE-cad-Z stents group was four times more than that of the BMS group, which implied that VE-cad-Z stents captured far more OECs than BMS. Moreover, DAPI and calcein staining confirmed the excellent biocompatibility of VE-cad-Z stents. Therefore, our present data provided strong evidence that VE-cadherin may be a promising eluting layer for promoting endothelium healing. This rapid re-endothelial ability is believed to reduce neointimal hyperplasia and thrombosis in long term outcomes²³⁾.

In addition to its remarkable performance *in vitro*, VE-cad-Z stents showed its advantages *in vivo* as well. Three BMS were found occluded by thrombus within 3 days after implantation, whereas all VE-cad-Z stents remained patent until harvest. We hypothesized that both excellent biocompatibility of zwitterionic stent and impressive OEC-capture ability of VE-cadherin contributed toward reduced *in vivo* thrombogenicity and improved the early patency rate of VE-

Fig. 9. Protein expression of stents 30 days after implantation.

A, Photographs of immunoblots of tissue from normal coronary (NCA), BMS, and VE-cad-Z stents. B, Densitometric analysis of protein expression (n=10 each). For statistical analysis, one-way ANOVA was performed. * $P < 0.05$ between two groups, ** $P < 0.01$ between two groups. NS=not significant.

cad-Z stents. *In vivo*, VE-cad-Z stents showed excellent anti-platelet ability, whereas its ability to capture OECs was comparatively unsatisfying. VE-cad-Z stents failed to capture more OECs than BMS at 3 days after implantation. Therefore, we hypothesized that VE-cadherin antibody was not specific to rabbit OECs, and this limited its ability to capture rabbit OECs. However, after 30 days of implantation, a confluent layer of OECs covered the luminal surface of VE-cad-Z stents. We hypothesized that specific platelet-resistant structure of PEG-MPC zwitterionic polymers as described above reduced the *in vivo* thrombogenicity and improved the early patency rate of VE-

cad-Z stents, and the compliance of the seaweed-like structure prolonged the collision time, reduced the recoil force, and helped VE-cadherin capture OECs in blood when systole or blood lashed against VE-cad-Z stents, which provided time window for VE-cad-Z stents to re-endothelialization.

Apart from thrombosis, another side effect is instant restenosis (ISR), which is because of uncontrolled neointimal hyperplasia and tissue proliferation in response to vessel injury³¹. Three and 30 days after stenting, histological and morphometric analysis demonstrated that employment of VE-cad-Z stents could significantly reduce neointimal formation and ISR. In addition, it is found that VE-cad-Z stents expressed a much higher level of eNOS protein and a much lower level of MCP-1 and α -SM Actin protein than BMS, and the expression of these proteins of the VE-cad-Z stents was comparable to the native carotid artery. It is well known that MCP-1 exhibits its most potent chemotactic activity toward monocytes and T lymphocytes³², α -SM Actin is present in high amounts in vascular SMC, and eNOS expression is relatively restricted to the vascular endothelium (the cell-specific expression of endothelial nitric-oxide synthase). Moreover, it is reported that the expression of eNOS is critical for functional DESs, which catalyzes the production of NO, a potent vasodilator that inhibits SMCs proliferation and migration, which are responsible for intimal hyperplasia as well as platelet aggregation and adhesion^{33, 34}. Therefore, we hypothesized that lack of functional endothelium and subsequent inflammatory cell infiltration into the injured artery caused smooth muscle cell migration and proliferation, which resulted in neointimal hyperplasia³⁰. Moreover, the neointimal area and ISR of BMS significantly increased with implantation time, whereas those of VE-cad-Z stents did not increase. We hypothesized that it may be because of the confluent layer of OECs that inhibit SMC migration and proliferation by a paracrine pathway³⁵ and thus prevented the neointimal hyperplasia. Therefore, complete re-endothelialization within a few days played an important role in the prevention of stent thrombosis and in the inhibition of neointimal growth¹³.

One limitation was that we did not perform the immunohistochemical analysis of the neointimal area; thus, evaluation of macrophage infiltration and degree of proliferative activities in the neointima was not available. Another limitation was that our study was limited to observations in experimental animal models; therefore, its clinical relevance to human being was uncertain.

Conclusion

Coating VE-cadherin onto zwitterionic stents may be an attractive approach for drug-eluting stents, and VE-cadherin coated zwitterionic stents seemed capable of capturing circulating OECs and endothelial cells effectively and specifically without the activation of platelets or recruitment of inflammatory cells and vascular SMC. To the best of our knowledge, this is the first report on DES constructed by coating VE-cadherin onto zwitterionic stents, and our findings provided strong evidence for the validity of our protocol. However, this is just a preliminary study of VE-cad-Z stents, and many questions are still unanswered. Therefore, we are going to further study the long term outcome and side effect of our novel DES.

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COI

No competing financial interests exist.

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