Study of novel coating strategy for coronary stents: simutaneous coating of VEGF and anti-CD34 antibody

Estudo da nova estratégia de revestimento para stents coronários: revestimento simultâneo de VEGF e anticorpo anti-CD34

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Abstract

Introduction: **Intravascular coronary stenting has been used in the treatment of coronary artery disease (CAD), with a major limitation of in-stent restenosis (ISR). The 316 stainless steel has been widely used for coronary stents. In this study, we developed a novel coating method to reduce ISR by simultaneously coating vascular endothelial growth factor (VEGF) and anti-CD34 antibody on 316L stainless steel.**

Methods: **Round 316L stainless steel sheets in the D-H group were polymerized with compounds generated from condensation reaction of dopamine and heparin using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS). Sixteen sheets from the D-H group were further immersed into 1ug/ml $VEGF_{165}$ and 3mg/ml heparin sodium one after another for 10 times, and named as the D-$(H\text{-}V)_{10}$ group. Eight sheets from the D-$(H\text{-}V)_{10}$ group were coated with anti-CD34 antibody and termed as the D-$(H\text{-}V)_{10}$-A group. Immunofluorescence assay and ELISA were used to evaluate whether the 316L stainless steel disks were successfully coated with VEGF and anti-CD34 antibody.**

Results: **The results of immunofluorescence assay and ELISA showed that VEGF could be detected in the D-$(H\text{-}V)_{10}$ and D-$(H\text{-}V)_{10}$-A group, suggesting the steel sheets were successfully covered with VEGF. Anti-CD34 antibody could only be observed in the D-$(H\text{-}V)_{10}$-A group, which was the only group coated with CD34 antibody. Both results suggested that the 316L stainless steel sheets were successfully coated with VEGF and anti-CD34 antibody.**

Conclusion: **Our study developed a method to simultaneously coat VEGF and anti-CD34 antibody to stainless metal steel. This research serves as a fundamental role for a novel coating strategy.**

Descriptors: **Coronary Artery Disease. Drug-Eluting Stents. Coronary Restenosis. Vascular Endothelial Growth Factor. Antigens, CD34.**

Resumo

Introdução: **O stent coronário intravascular tem sido utilizado no tratamento de doença arterial coronária, com uma maior limitação de restenose intra-stent (RIS). O aço inoxidável**

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Abbreviations, acronyms & symbols	
BSA	Bovine serum albumin
CAD	Coronary artery disease
DESs	Drug eluting stents
EDC	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide
ISR	In-stent restenosis
NHS	N-hydroxysuccinimide
VEGF	Vascular endothelial growth factor

316 tem sido amplamente utilizado para stents. Neste estudo, foi desenvolvido um novo método de revestimento para reduzir a RIS para revestir simultaneamente o fator de crescimento endotelial vascular (VEGF) e anti-CD34 em aço inoxidável 316L.

Métodos: **Placas de aço inoxidável 316L redondas no grupo DH foram polimerizadas com compostos gerados a partir da reacção de condensação de dopamina e heparina utilizando N- (3-dimetilaminopropil) -N'-etilcarbodiimida (EDC) e N-hidroxissuccinimida (NHS). Dezesseis folhas a partir do grupo DH foram ainda imersas em 1 ug/ml de VEGF 165 e 3 mg/ml de heparina sódica, um após outro por 10 vezes, sendo denominado como o grupo D-$(HV)_{10}$. Oito folhas de D-$(HV)_{10}$ foram revestidas com anticorpo anti-CD34 e denominado como grupo D-$(HV)_{10}$-A. Testes de imunofluorescência e ELISA foram usados para avaliar se os discos de aço inoxidável 316L foram revestidos com sucesso com VEGF e anticorpo anti-CD34.**

Resultados: **Os resultados dos testes de imunofluorescência e ELISA mostraram que o VEGF pôde ser detectado nos grupos D-$(HV)_{10}$ e D-$(HV)_{10}$-A, evidenciando que as chapas de aço foram cobertas com VEGF com sucesso. O anticorpo anti-CD34 podia apenas ser observado no grupo D-$(HV)_{10}$-A, o único grupo revestido com anticorpo CD34. Ambos os resultados sugerem que as chapas de aço inoxidável 316L foram revestidas com sucesso com VEGF e anticorpo anti-CD34.**

Conclusão: **Nosso estudo desenvolveu um método para revestir simultaneamente VEGF e anti-CD34 de aço inoxidável. Esta pesquisa tem um papel fundamental para a nova estratégia de revestimento.**

Descritores: **Doença Arterial Coronariana. Stents Farmacológicos. Reestenose Coronária. Fator de Crescimento Endotelial Vascular. Antígenos CD 34.**

INTRODUCTION

In-stent restenosis (ISR) was mainly caused by complications of intracoronary stent placement, including thromboembolic events and neointimal hyperplasia due to smooth muscle cell hyperproliferation. Drug eluting stents (DESs) have been designed mainly to reduce cellular proliferation and thus reduce ISR. Drug-eluting stents currently on the market release cytotoxic drugs such as paclitaxel and rapamycin to inhibit neointimal hyperplasia at the expense of delaying endothelialization[1,2].However, the incomplete endothelialization of the stent surface has been suggested that may lead to the increased long-term incidence of thrombosis and ISR[3].The critical role of the vascular endothelium in preventing thrombosis and regulating neointimal hyperplasia has resulted in restenosis prevention strategies that focus on enhancing endothelialiazation[4-6].

Vascular epithelial growth factor (VEGF), a cytokine originally described in 1983[7], is involved in processes essential to the growth, maintenance and repair of vascular structures. Exogenous VEGF has been reported to show accelerated re-endothelialization of damaged arteries in the rat carotid artery and attenuated intimal hyperplasia[8]. The delivery of VEGF using VEGF-eluting stents showed that it has been used to promote revascularization and re-endothelialization by stimulating endothelial progenitor cell migration and maturation [9,10]. Circulating endothelial progenitor cells (EPCs), a subset of bone marrow-derived stem cells, possess the ability to differentiate into functional and mature endothelial cells and recently have been identified as a key factor for re-endothelialization[11]. The EPC capture stents have been developed using immobilized antibodies targeted at EPC surface antigens, such as CD34[12].

In this report, to further accelerate re-endothelialization, we aimed to develop method to simultaneously coat VEGF and anti-CD34 antibody. Our results showed that VEGF and anti-CD34 antibody were successfully coated onto the 316 stainless steel.

Experimental procedures

Preparation of the coated steel sheet

Round 316L stainless steel sheets (diameter 6 mm, thickness 1 mm) were used to facilitate the measurement and evaluation of the properties of the coating, instead of bare metal stents with limited testable aspects and relatively high costs. The metal surface was polished, washed and dried at 60ºC for 24 hours, then sterilized by ultraviolet radiation. Heparin was conjugated to dopamine using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS)[13].The sterilized sheet was polymerized with compounds generated from condensation reaction of dopamine (Aladdin, Shanghai, China) and heparin (Aladdin) as described[13], and termed as the D-H group.

Then, sixteen sheets from the D-H group were immersed into 1ug/ml $VEGF_{165}$ (Life technologies) dissolved in phosphate-buffered saline (PBS, PH 7.4) for 30 min at room temperature (RT). Washed with Milli-Q water for 3 times (5 min per time) and dried under nitrogen. Then, a Milli-Q water solution of heparin sodium (3mg/ml) was subsequently dip-coated for 30 min at RT. Washed with Milli-Q water for 3

times (5 min per time) and dried under nitrogen. These coating procedure were repeated 10 times, and these sixteen steel disks were set as the D-$(H\text{-}V)_{10}$ group.

The D-$(H\text{-}V)_{10}$-A group was obtained by the following procedures. Eight steel disks from the D-$(H\text{-}V)_{10}$ group were immersed into 0.1mg/ml protein A (Aladdin) dissolved in PBS for 30 min at RT, blocked in 10 mg/ml bovine serum albumin (BSA; BD Biosciences) for 24 hours at 4 °C and immersed with 2 μg/ml rabbit anti-human CD34 antibody (Abcam, Cambridge, UK) for 24 hours at 4 °C. After the whole procedure, three groups of sheets were obtained (8 sheets/each group) for further evaluations.

Immunofluorescence detection of the coated VEGF and anti-CD34 antibody

The levels of coated VEGF and anti-CD34 antibody of the stainless steel sheets were detected using immunofluorescence microscopy in the D-H group, D-$(H\text{-}V)_{10}$ group and D-$(H\text{-}V)_{10}$-A group. The sheets were soaked for 1 hour in blocking solution (PBS containing 3% BSA). For the detection of VEGF, all the three groups were incubated with rabbit anti-human VEGF primary antibodies (1:100; Abcam) overnight at 4 °C, washed three times with PBS, and then incubated with Alexa Fluor® 488 Goat Anti-Rabbit IgG (Life technologies) at room temperature for 1 hour. For the detection of rabbit anti-human CD34 antibody, secondary antibodies- Alexa Fluor® 488 Goat Anti-Rabbit IgG were directly incubated. Wash three times to remove non-specific binding of the secondary antibodies and observe using a Laser scanning confocal microscope (Leica TCS SP5; Leica Microsystems, Germany).

The detection of coated VEGF and anti-CD34 antibody by ELISA

Coated steel sheets from the D-H group, D-$(H\text{-}V)_{10}$ group and D-$(H\text{-}V)_{10}$-A group were immersed into RIPA lysis buffer for 24 hours at 4 °C to dissolve VEGF and anti-CD34 antibody. The levels of VEGF and anti-CD34 antibody were detected using $VEGF_{165}$ ELISA Kit (Life technologies) and Rabbit IgG ELISA Kit (Novus Biologicals, USA), respectively.

Statistical analysis

Statistical significance was evaluated by comparing mean values (±standard deviation) using the two-tailed Student's t-test for independent groups. The probability value $P<0.05$ was considered to be statistically significant.

RESULTS

Immunofluorescence staining

To evaluate whether the 316L stainless steel sheets were successfully coated with VEGF and anti-CD34 antibody, immunofluorescence assay was performed. The results of immunofluorescence assay showed that VEGF could be detected in the D-$(H\text{-}V)_{10}$ and D-$(H\text{-}V)_{10}$-A group, suggesting the steel sheets were successfully covered with VEGF. Anti-CD34 antibody could only be observed in the D-$(H\text{-}V)_{10}$-A group, which was the only group that coated with CD34 antibody (Figure 1). Our findings suggested that the 316L stainless steel sheets were successfully coated with VEGF and anti-CD34 antibody.

ELISA detection of coated VEGF and anti-CD34 antibody

To further evaluate the coverage of VEGF and anti-CD34 antibody, ELISA was performed in the D-H group, D-$(H\text{-}V)_{10}$ group and D-$(H\text{-}V)_{10}$-A group. Similar to the results of immunofluorescence assay, VEGF could be detected in the D-$(H\text{-}V)_{10}$ and D-$(H\text{-}V)_{10}$-A group, and anti-CD34 antibody could only be observed in the D-$(H\text{-}V)_{10}$-A group (Figure 2). The results of ELISA further confirmed that we successfully coated VEGF and anti-CD34 antibody onto the 316 stainless steel.

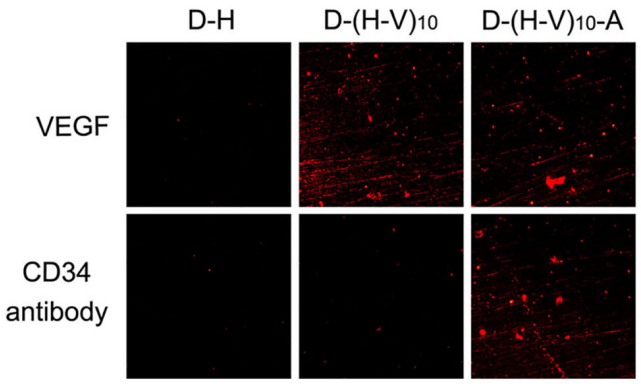

Fig. 1 - Immunofluorescence detection of the coated VEGF and anti-CD34 antibody. The levels of coated VEGF and anti-CD34 antibody of the 316L stainless steel were detected using immunofluorescence microscopy in the D-H group, D-$(H\text{-}V)_{10}$ group and D-$(H\text{-}V)_{10}$-A group.

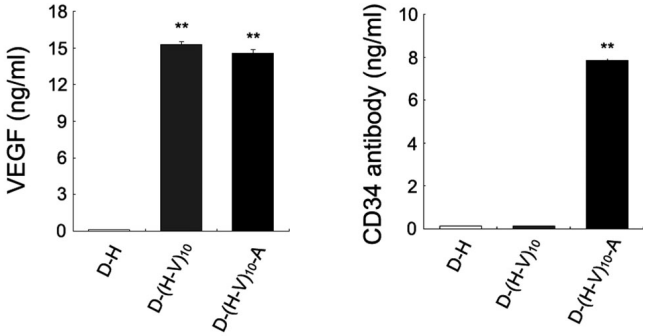

*Fig. 2 - Detection of the coated VEGF and anti-CD34 antibody by ELISA. The results of detection of coated VEGF (left) and anti-CD34 antibody (right) by ELISA. Data are reported as mean ± SD for three independent experiments. Statistically significant differences are indicated as **P<0.01, student's* t*-test.*

DISCUSSION

Intravascular coronary stenting has been widely used for many years, and it has increased the quality of life and life expectancy of patients with coronary disease. The 316 stainless steel is one of the most widely used materials for coronary stents with a board range of mechanical properties. However, the exposure of flowing blood to the bare metal stent may lead to thrombus formation and smooth muscle cell proliferation, and finally cause in-stent restenosis (ISR).Therefore, huge amount of recent work has attempted to develop non-thrombogenic coating for these metallic stents[14-16].

Early drug-eluting stents coating with various kind of drugs have been designed to reduce the restenosis through minimize vascular inflammation and cellular proliferation[17], which including a polymer-based drug delivery platform and a pharmacologic agent (usually an immunosuppressant and/or antiproliferative compound).Though early trials seem to be exciting with markedly reduction rates of ISR (5%-8%) [18,19], long-term follow-up studies showed that DESs implantation increased the long-term risk of thrombosis by 15% -35% compared with bare-metal stents implantation[20].

Based on our expanding understanding of pathophysiology of restenosis, novel stent coating strategies have been developed, such as delivery of VEGF (e.g., VEGF-eluting stents[9]) and the use of antibodies that recognize epitopes specific to endothelial progenitor cells (e.g., anti-CD34-coated stents[12]). Both coating strategies have been designed to inhibit thrombosis mainly through promoting re-endothelialization of cardiovascular stents. Many clinical studies suggest that the Genous EPC-capture stent is a safe choice for patients with coronary disease[21-24]. However, Adrian et al.[25] reported that a similar late luminal loss of Genous EPC-capture stent to that of a bare-metal stent, despite initial optimism of rapid endothelialization.

In this study, our approach of surface modification has included the combination of VEGF and anti-CD34 antibody. The goal of this combination is to further accelerate endothelial repair, and thus further reduce the exposure time of stents in blood, decrease the rate of long-term thrombosis and shorten the time of antiplatelet therapy for patients. We firstly used dopamine-mediated heparin coating[13] and then a layer-by-layer method was employed to build multilayer films composed of heparin and VEGF on metal substrates. Based on the specific affinity of protein A and IgG antibodies, the protein A allows the subsequent immobilization of the anti-CD34 antibody. Our primary results of immunofluorescence and ELISA showed that the stainless metal steel was successfully coated with VEGF and anti-CD34 antibody (Figures 1 and 2). This research serves as a fundamental role for the novel coating strategy of simultaneous coating of VEGF and anti-CD34 antibody and further studies on the toxicity and effect of the combined coating are currently ongoing.

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Authors' roles & responsibilities	
CLS	Analysis and interpretation of data; statistical analysis; design and study design; carried out operations and experiments
QL	Analysis and interpretation of data; statistical analysis; carried out operations and experiments
YPY	Analysis and interpretation of data; statistical analysis; carried out operations and experiments
GW	Analysis and interpretation of data; statistical analysis; carried out operations and experiments
JPW	Analysis and interpretation of data; statistical analysis; carried out operations and experiments
YL	Analysis and interpretation of data; statistical analysis; carried out operations and experiments
JCZ	Analysis and interpretation of data; statistical analysis; carried out operations and experiments
HYD	Analysis and interpretation of data; carried out operations and experiments
JGL	Analysis and interpretation of data; carried out operations and experiments
YHL	Analysis and interpretation of data; carried out operations and experiments
JL	Analysis and interpretation of data; carried out operations and experiments
YL	Analysis and interpretation of data; carried out operations and experiments
DC	Analysis and interpretation of data; carried out operations and experiments
BL	Analysis and interpretation of data; carried out operations and experiments

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