The short-term effects on restenosis and thrombosis of echinomycin-eluting stents topcoated with a hydrophobic heparin-containing polymer

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Abstract

Although drug-eluting stents (DESs) have become the most effective means of treating coronary artery disease, safety concerns regarding their thrombogenicities remain to be surmounted. Here, we report on a novel type of DES capable of preventing restenosis and thrombosis. The DES was prepared by coating a bare metal stent with echinomycin (an anti-proliferative drug) in polyurethane by a spray drying method. Hydrophobic heparinized polymer was then topcoated onto stent over echinomycin/PU layer by dipping to improve hemocompatibility. The two-layered stent was characterized regarding surface and cross-sectional morphology, drug release pattern, platelet adhesion in vitro, and restenosis in vivo. It was found that the heparin topcoat acts as a diffusion barrier that allows the controlled release of drug in a sustained manner. Also, the heparin coated layer effectively reduced platelet adhesion, indicating excellent hemocompatibility. From the animal test using pigs, it was evident that the developed DESs can minimize neointimal proliferation and thrombus formation. The devised hydrophobic heparinized polymer-coated DES effectively reduced both restenosis and thrombosis, suggesting that they have potential as tools for the treatment of coronary artery diseases.

Keywords: Drug-eluting stent; Heparinized polymer; Echinomycin; Restenosis; Thrombosis

1. Introduction

Stents have been used to maintain arterial patency after balloon angioplasty in patients that have undergone a percutaneous coronary intervention (PCI). Moreover, since PCI can cause injury to blood vessels, in particular, it causes neointimal hyperplasia which may result in vessel occlusion, a process known as in-stent restenosis [1–3]. It has been reported that the rate of restenosis is dependant on various parameters, such as, vessel size, diabetes mellitus, stent type, and others [4–6]. Moreover, recent advances in coronary stent technology have produced many different types of stents that show reduced levels of restenosis [6]. In particular, the concept of incorporating a therapeutic agent into the vessel wall has emerged as an attractive solution to surmount the problem of restenosis. Several kinds of drug-eluting stents (DESs) have been developed using stainless steel coated with different polymeric matrices containing drugs, and a number of these are clinically available [7–11]. These DESs have been designed to release a controlled amount of antiproliferative drugs that can reduce smooth muscle cell growth and
prevent inflammatory response. Sirolimus-eluting (Cypher®, Cordis) and paclitaxel-eluting stents (Taxus®, Boston Scientific) have been most extensively studied and their use has markedly altered the outcome of patients that have undergone coronary angioplasty, primarily because they reduce restenosis [12–14].

In addition to the prevention of neointimal formation and resistance to thrombus formation are also important requirements. Stent thrombosis is not a rare and potentially life-threatening complication of PCI [15–18]. Recently concerns regarding the potential thrombogenicities of stents have increased because of a flurry of stent thrombosis reports for the Cypher® stent [16]. Therefore, the selection of noninflammatory and nonthrombogenic coating materials should play a vital role in the successful use of stents. The Cypher® stent employs a mixture of polyethylene-co-vinyl acetate and poly(n-butyl methacrylate), whereas Taxus® uses poly(styrene-b-isobutylene-b-styrene) triblock copolymer as a coating material [12,19]. It should be noted that although such polymers can control the release rates of incorporated drugs, their biocompatibilities remain unclear. Many approaches have been used to generate a modified stent surface by chemically grafting or physically anchoring hydrophilic polymers, such as, heparin [20–22] and phosphorylcholine-containing polymers [23].

Here, we report a novel type of DES capable of releasing an antiproliferative drug in a sustained manner. This DES was developed in an attempt to minimize restenosis and remove the possibility of thrombus formation on stent surfaces. First, we prepared a hydrophobic heparinized polymer which can be physically retained on a hydrophobic surface for a long time of period. Second, the stent surface was precoated with polyurethane (PU) containing the antiproliferative drug, echinomycin. Finally, the stent surface was topcoated with the heparin layer using a simple dipping method. The resulting two-layered stent was evaluated with regard to drug release behavior, platelet adhesion and the prevention of restenosis in pigs.

Echinomycin is a bifunctional intercalating agent derived from Streptomyces echinatus and is a potent anticancer agent [24]. It binds strongly to double-stranded DNA and acts as a molecular staple, sandwiching two base pairs within its U-shaped conformation. Moreover, animal studies suggested that echinomycin has beneficial effects on vessel response to injury and smooth muscle cell proliferation [25,26].

2. Experiments

2.1. Synthesis of heparinized polymer for topcoating

The hydrophobic heparinized polymer was prepared using the method described in US patent no. 6702850 B1. Briefly, carboxyl group of polyacrylic acid (PAA, MW: 15,000, 0.67 mmol, Sigma, St. Louis, MO) was activated using N,N-dicyclohexylcarbodiimide (DCC, 110 mmol, Sigma Chemical Co.) and N-hydroxysuccinimide (NHS, 151 mmol, Sigma) in DMF at 4 °C for overnight. The residual product, dicyclohexylurea (DCU) was removed by filtration and the produced polyacrylic acid-N-hydroxysuccinimide (PAA-NHS) was purified by recrystallization and precipitation in methanol solution. Both PAA-NHS and heparin (MW: 12,000, 0.1 mmol, Pharmacia Hepar Co., Franklin, OH) were reacted at room temperature for 1 h, and then octadecylamine (ODA, 53.8 mmol, Sigma) in THF was added. After 15 h reaction, the reacted product was centrifuged at 8000 rpm for 30 min, following the precipitate was dispersed in water. The precipitate in water was evaporated off using a rotary evaporator, and dried under vacuum. To obtain a fine product, it was dispersed in cyclohexane for 30 min, and then, centrifuged at 8000 rpm for 30 min. The supernatant was added to acetone and then stirred for 1 h. The solution was centrifuged and the precipitate (final product) was dried under vacuum oven for 2 days. The final product was analyzed using FT-IR (Spectrum 2000, Perkin Elmer, Newcastle Upon Tyne, UK), ¹H-NMR (JEOL JNM-LA 300WB FT-NMR, Tokyo, Japan), Element analysis (ECS 4010 Costech Analytical Technologies Inc., Valencia, CA) and Light Scattering (S4700, Malvern Instruments Ltd., Worcestershire, UK).

2.2. Preparation of the two-layered stent

In order to prepare the basecoating solution, PU (Pellethane®, Dow chemical Co., Midland, MI) was dissolved in tetrahydrofuran at a concentration of 3 wt%, and echinomycin (Sigma) was added to give a solution concentration in the range of 0–5 wt%. This PU/drug solution was then stored at 4 °C, prior to the use. Bare metal stents were washed with methanol and dried in a vacuum at room temperature for 1 day. The basecoating solution was sprayed onto the bare stent for 10 min and then dried in a vacuum at room temperature. This process was repeated five times. The topcoat was applied by a simple dipping method, in which the coating solution was prepared by dissolving hydrophobic heparinized polymer in cyclohexane at a concentration of 1–5 wt%. To evaluate the finished surfaces and cross-sectional morphologies, stents were observed under a scanning electron microscope (SEM, JSM-6700F, JEOL, Tokyo, Japan).

2.3. Platelet adhesion on the two-layered stent

The whole blood from a white rabbit (Korean Animal Center, Seoul, Korea) was carefully collected in a polypropylene syringe containing 3.8% sodium citrate solution. Platelet-rich plasma (PRP) containing 3 x 10⁵ cells/μl, measured using a Coulter counter (Model Z1, Coulter Electronics Inc., Hialeah, FL), was prepared by mixing PRP and platelet-poor plasma (PPP) that were obtained by centrifuging the whole blood for 15 min at 1200 and 2000 rpm, respectively. The two-layered stents were immersed into 5 ml of PRP pre-warmed to 37 °C. After 1 h, they were gently rinsed with fresh phosphate-buffered saline (PBS, pH 7.4) to remove non-adherent platelets, fixed with 2.5% glutaraldehyde solution in PBS for 2 h at room temperature, dehydrated at 4 °C in a gradual ethanol/distilled water mixture from 50% to 100% in steps of 10% for 10 min each, and freeze-dried. The adherent platelets on stent surfaces were observed by SEM.

2.4. Elution echinomycin from the two-layered stents

The amount of echinomycin eluted from the basecoat (PU coating layer) was compared to that from a two-layered stent. Both stents were incubated separately in PBS (pH 7.4) at 37 °C. The amounts of echinomycin that eluted were determined over a 9-day period and were measured as follows: the eluted echinomycin in buffer was extracted using dichloromethane via strong agitation for 15 s, and dried in vacuum at room temperature for 1 day. Echinomycin was then dissolved in acetonitrile and its concentrations were determined by HPLC (Shimadzu, Tokyo, Japan).
2.5. Evaluation of restenosis in vivo

Pigs (weight 23–25 kg, Daehan Biolink Ltd., Korea) were used to evaluate the effect of two-layered stents deployment on restenosis. All pigs were kept under the same condition and were provided with an experimental feed devoid of lipids. The pigs were also administered 300 mg/day of aspirin in feed before experiment. For the animal test, bare and single-layered PU-coated stents were compared with the two-layered stents containing different amounts of echinomycin. All the animal experiments were carried out in accordance with the procedures outlined in the following figures:

Fig. 1. Schematic illustration of a two-layered stent. (a) Chemical modification of heparin with PAA and ODA and (b) structure of a two-layered stent.

Fig. 2. SEM observation of surfaces of stents. (a) A bare stent, (b) a base-coated stent (echinomycin), (c) a top-coated stent (hydrophobic heparinized polymer), and (d) a top-coated stent after drug released.
Each pig was anesthetized using an injection of ketamine (22 mg/kg). Thereafter, an incision was made in the front of the neck at the midline to expose the carotid artery, followed by an injection of heparin (300 U/kg). A guide-wire (eight French) was then inserted into the carotid artery, and a guide catheter was maneuvered to the coronary artery. An appropriate site on the right coronary artery was selected for angiography.

A stent was attached to a balloon catheter capable of being expanded to 10–20% larger than the diameter of the coronary artery. The catheter was maneuvered to the selected site in the coronary artery and the balloon was inflated to its maximum size for 30 s at 12 atmospheres pressure to intentionally damage the coronary artery. It was then deflated and the catheter was withdrawn with the stent in position. To prevent the coronary artery contraction following the blood vessel damage, nitroglycerin (200 μg, Sigma) was administered. After the operation, coronary artery angiography was conducted to observe the degree of damage to the coronary artery and the potency of blood flow. The artery guide-wire was then removed and the slit in the carotid artery was closed.

After 28 days, animals were anesthetized and a guide-wire was inserted to confirm the potency of coronary artery. A lethal amount of pentobar and potassium chloride were injected via the guide catheter to induce euthanasia. The pig’s heart was removed through the thorax and subjected to a perfusion-fixation procedure and OEC (GE Medical, Los Angeles, CA) was used to determine the degree of damage to blood vessels. The damaged portion of the artery was removed from the heart. The specimen containing the stent was fixed using an Embedding System (Technovit 7100, Kulzer, Germany) and was sliced into sections using a microtome equipped with a tungsten blade. Sections were stained with hematoxylin-eosin and elastic Van Gieson. Morphological analysis of the slices was carried out under a microscope.

3. Results

The synthesized heparinized polymer used for top coating was analyzed using FT-IR, 1H-NMR, elemental analysis and light scattering to determine the chemical structure of the heparin-PAA-ODA derivative. From the results, the binding mole ratio of heparin/PAA/ODA was 2:3:510 and the weight-average molecular weight of the derivative was 509,000 Da (Fig. 1). The synthesized heparinized polymer used for top coating was analyzed using FT-IR, 1H-NMR, elemental analysis and light scattering to determine the chemical structure of the heparin-PAA-ODA derivative. From the results, the binding mole ratio of heparin/PAA/ODA was 2:3:510 and the weight-average molecular weight of the derivative was 509,000 Da (Fig. 1).

The surface morphologies of bare and coated stents were observed using SEM, as shown in Fig. 2. Compared to bare stents, all coated stents had smoother surfaces. However, there were no significant differences between the surfaces of
precoated and two-layered stents. The results implied that echinomycin/PU was homogeneously coated onto bare metal stents by the spray drying method, and that the hydrophobic heparinized polymer adequately coated onto the PU basecoat. It is important to note that the surfaces morphologies of two-layered stents were maintained even after the 9-day release experiments, indicating that the hydrophobic heparinized polymer topcoat was relatively unaffected by this treatment.

The cross-sectional morphologies of stents are shown in Fig. 3. These stents were precoated with a PU solution (containing 3 wt% in tetrahydrofuran) containing 5 wt% of echinomycin. After being dried under vacuum, they were further coated with a heparinized polymer solution (1–5 wt% in cyclohexane). It was evident that the heparinized polymer can be coated uniformly on the basecoat by simple dipping, applied in this study. The thickness of the basecoat, composed of PU and echinomycin, was 20–22 μm, whereas that of the topcoat, consisting of hydrophobic heparinized polymer, was dependant on the concentration of the heparinized polymer solution used: the thickness of the topcoat was correlated with the heparinized polymer concentration.

Fig. 4 shows the in vitro release pattern of echinomycin from coated stents. Echinomycin was released faster from precoated stents than from two-layered stents, although all stents released echinomycin in a sustained manner for 9 days. In addition, all stents showed a similar release pattern, i.e., rapid release over the first day, followed by slower release. The slower release of echinomycin from two-layered stents than from precoated stents, implies that the topcoat acts as a diffusion barrier and retards echinomycin release from the PU basecoat.

Platelet adhesion to stent surfaces is significantly related to the process of protein adsorption and thrombus formation. For platelet adhesion studies, we treated the surfaces of bare and coated stents with PRP separated from the blood of a healthy rabbit. Fig. 5 shows SEM images, after in vitro platelet adhesion tests. The surfaces of the bare stents were covered with numerous platelets, which were highly activated based on their morphologies such as pseudopodia. However, precoated and two-layered stents showed less platelet adhesion. In particular, only a few platelets were found on the surface of two-layered stents, which suggests that the topcoat layer consisting of hydrophobic heparinized polymer effectively reduces the platelet adhesion.

The effect of stent deployment on restenosis was evaluating using a porcine model (Fig. 6). All pigs survived until completion of the 4-week study without evidence of myocardial infarction on gross inspection. Four-week follow-up angiograms showed that echinomycin had a marked effect, and the largest reduction in neointimal hyperplasia was found at the highest echinomycin dose. In control angiograms of PU-coated stent, distinctive vessel narrowing was observed, though this narrowing was reduced at the intermediate doses (0.1% and 1% of echinomycin). The use of two-layered stents containing a 5% echinomycin basecoat remarkably reduced neointimal proliferation.

From the animal testing, intima-media ratios (IMRs) were estimated (Fig. 7). Of the stents applied in this study, the bare stent exhibited the highest I/M ratio, indicating significant smooth muscle cell proliferation. The two-layered stents loaded with echinomycin significantly reduced restenosis 28 days after balloon injury. In particular, the two-layered stent loaded with 5% echinomycin effectively inhibited intimal thickening and smooth muscle cell proliferation.

4. Discussion

In this study, we prepared stents coated with the two layers to inhibit restenosis and thrombosis due to the release of echinomycin over a sustained period of time and the conducting of a bifunctional stent. An important consideration in the design of the two-layered stent was the release rate of echinomycin from the two-layered stent. It is desirable that an effective therapeutic amount of echinomycin be released from the stent over a long period of time without burst release. A high release rate immediately following implantation is undesirable, and could possibly induce local toxicity. The basecoat layer was prepared using solutions containing PU and echinomycin in THF, which were sprayed onto the stents to produce homogeneous and smooth surfaces. The topcoat layer consisted of an anti-thrombogenic, heparinized polymer which was applied by dipping process. The introduction of the
topcoat effectively reduced or prevented the burst release of echinomycin, thereby allowing sustained release over 28 days. When the heparin molecule is modified using a hydrophobic agent, it is essential that the active sequence responsible for anti-thrombogenic activity remains unaltered. We previously confirmed that the hydrophobic heparin used retains high bioactivity [27]. In terms of the synthesis of hydrophobic heparinized polymer, the carboxyl group of PAA is coupled with the amine groups of heparin. If other functional groups of heparin, such as, sulfonyl, carboxyl, or hydroxyl groups, had been used in the coupling reaction in order to increase the molar binding ratio of PAA to heparin, the activity of the heparin derivatives would have completely disappeared. This is because there are sulfonyl, carboxyl, and hydroxyl groups at the active site of heparin, and conjugated PAA can block these sites of heparin. However, since there are no amine groups in the active site of heparin, only the amine group of heparin is available for the conjugation. Therefore, we note that the hydrophobic heparinized polymer actively prevents blood coagulation around the stent, thus inhibiting thrombosis and subacute stent thrombosis. In particular, since the heparinized polymer could make a thin film and completely cover the stent surface without any defects, it could prevent thrombosis more effectively. Furthermore, the top coated hydrophobic heparinized polymer reduces or prevents burst release of echinomycin from the basecoat layer in the long term.

Bare stents were associated with an increased intimal thickness, persistent intimal fibrin deposition, intraintimal hemorrhage, and increased intimal and adventitial inflammation. On the other hand, the coated stents, showed minimal thickening, thus demonstrating the effect of the echinomycin. Based on our findings, 30% of echinomycin in the two-layered stent was eluted during the first 24 h. This release rate was dependent on the thickness and concentration of hydrophobic heparinized polymer layer (topcoat) which allowed a proper antithrombotic effect after stent implantation. Moreover, echinomycin was released at pharmacologically active levels for 4 weeks.

Fig. 5. Platelet adhesion of (a) a bare stent, (b) a base-coated stent and (c) a top-coated stent. Lower magnification (100 x or 200 x ) was used to visualize more cell population, and then changed to higher magnification (3000 x ) for more detail images.
Strong evidence has been presented in the animal tests that neointimal hyperplasia and restenosis are linked to platelet adhesion, aggregation, and thrombus formation.

5. Conclusion

In this study, the two-layered stents, composed of a PU/echinomycin basecoat and a hydrophobic heparin topcoat, were successfully prepared. They were found to release echinomycin in a sustained manner at least for 9 days, and to efficiently prevent platelet adhesion. Animal testing confirmed that the two-layered stents effectively inhibited smooth muscle cell proliferation and restenosis. Overall, we believe that the heparinized polymer-coated DESs developed in this study might be useful for the treatment of coronary artery disease.

References


