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Acceleration of re-endothelialization and inhibition of neointimal formation using hybrid biodegradable nanofibrous rosuvastatin-loaded stents

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ABSTRACT

Incomplete endothelialization and neointimal hyperplasia of injured arteries can cause acute and late stent thromboses. This work develops hybrid stent/biodegradable nanofibers for the local and sustained delivery of rosuvastatin to denuded artery walls. Biodegradable nanofibers were firstly prepared by dissolving poly(D,L)-lactide-*co*-glycolide and rosuvastatin in 1,1,1,3,3,3-hexafluoro-2-propanol. The solution was then electrospun into nanofibrous tubes, which were mounted onto commercially available bare-metal stents. The *in vitro* release rates of the pharmaceuticals from the nanofibers were determined using an elution method and a high-performance liquid chromatography assay. The experimental results thus obtained suggest that the biodegradable nanofibers released high concentrations of rosuvastatin for four weeks. The effectiveness of the local delivery of rosuvastatin in reducing platelets was studied. The tissue inflammatory reaction caused by the hybrid stents that were used to treat diseased arteries was also documented. The proposed hybrid stent/biodegradable rosuvastatin-loaded nanofibers contributed substantially to the local and sustainable delivery of a high concentration of drugs to promote re-endothelialization, improve endothelial function, reduce inflammatory reaction, and inhibit neointimal formation of the injured artery. The results of this work provide insight into how patients with a high risk of stent restenosis should be treated for accelerating re-endothelialization and inhibiting neointimal hyperplasia.

1. Introduction

Percutaneous coronary interventions (PCI) are made using balloon-expandable stents to treat coronary artery disease (CAD) worldwide. Such treatment provides better acute and chronic outcomes than conventional balloon angioplasty [1]. However, injury-induced migration and proliferation of smooth muscle cells (SMCs) remains the major pathophysiological cause of neointima formation and subsequent in-stent restenosis [2]. The use of drug-eluting stents (DESs) that are coated with either sirolimus or paclitaxel can greatly reduce the rate of stent restenosis [3,4]. These compounds, however, markedly inhibit endothelial cell proliferation and delay re-endothelialization, resulting in acute stent thrombosis [5]. Additionally, hypersensitivity to metallic alloy or the non-degradable polymer coating on a bare-metal stent (BMS) scaffold substantially accelerates the development of late stent thrombosis [6]. Hence, a DES that sustainably inhibits SMC growth without significantly interfering with post-procedural endothelial proliferation is highly desired [7].

Statins are a class of drugs that reduce cholesterol levels by inhibiting the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which has crucial role in the production of cholesterol. The uptake of modified low-density lipoprotein (LDL) cholesterol, which contributes to the lipid core of the plaque, has as an important role in forming atherosclerotic plaque in patients

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with or without CAD [8–10]. Statins also exhibit non-cholesterol-dependent pleiotropic properties, inhibiting the proliferation of SMC and platelet activation, and promoting both endothelial function and vascular inflammation [11].

Local delivery of statins to the diseased site through stents might provide the advantage of delivering high drug concentrations to the diseased site, minimizing possible systemic side effects, including a mildly increased risk of diabetes, abnormalities in liver enzymes and such rare but severe effects as muscle damage. Despite the fact that previous studies have established the safety of statin-eluting stents, they have failed to provide evidence of low-than-expected neointimal inhibition perhaps owing to low efficacy, weak potency, and insufficient drug loads [12,13].

This work develops poly(D,L)-lactide-*co*-glycolide (PLGA) nanofiber-loaded stents that locally deliver sustainable various doses of statins to injured arterial walls. Rosuvastatins, a highly potent HMG-CoA reductase inhibitor that exhibits excellent pharmacologic characteristics [14], improves endothelial function, and has both anti-proliferative and anti-inflammation properties, was used herein. PLGA is a member of the class of synthesized biodegradable and non-cytotoxic copolymers, and it can be absorbed over time without accumulating in the vital organs [15,16]. Following its introduction into the human body, PLGA triggers a minimal inflammatory response and is degraded by the hydrolysis of its ester linkages to form lactic and glycolic acids [17]. This process reduces the risk associated with the long-term presence of durable polymers in the arterial vessel wall [18–20]. The *in vitro* release rates of the pharmaceuticals from the nanofiber-loaded DES were evaluated using an elution method and high-performance liquid chromatography (HPLC) assay. The effectiveness of the local delivery of rosuvastatin in reducing platelets was studied. The inflammatory reaction of the tissue that was caused by the hybrid stents in the treatment of diseased arteries was also investigated. The effect of drug loading on the acceleration of endothelial cell regrowth as well as functionally recovery, and the inhibition of neointima formation was examined using an experimental animal model of vascular injury and stenting.

2. Materials and method

2.1. Fabrication of rosuvastatin-loaded nanofibrous tubes

The PLGA used herein is commercially available (Resomer RG 503, Boehringer, Germany); has a lactide:glycolide ratio of 50:50, and has a molecular weight of approximately 33,000 Da, as determined using a Gel permeation chromatograph that was equipped with a Waters 2414 refractive index detector. Rosuvastatin and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) were purchased from AstraZeneca U.K. Limited (London, U.K.) and Sigma–Aldrich (Saint Louis, MO, U.S.A.) respectively. The electrospinning setup in this investigation involved a syringe and needle with an internal diameter of 0.42 mm, a ground electrode, a metallic pin (with a diameter of 0.95 mm) mounted on a motor, and a high-voltage supply [21]. The needle and the metallic pin were connected to the high-voltage supply, which produced positive direct current voltages and currents of up to 35 kV and 4.16 mA, respectively. The rate of rotation of the motor was 300 rpm. To electrospin the nanofibers, PLGA and rosuvastatin (280 mg/10 mg, w/w) in a pre-set weight ratio was firstly dissolved into 1 ml of HFIP. The solution was then electrospun using a syringe pump with a volumetric flow rate of 3.6 ml/h, to yield nanofibrous tubes on the metallic pin. The distance between the needle tip and the ground electrode was 10 cm, and a positive voltage of 17 kV was applied to the polymer solutions. All electrospinning experiments were conducted at room temperature. After electrospinning, the electrospun nanofibrous tube was hand-crimped on the outside of a commercially available Liberté BMS (balloon-expandable 316L stainless steel) with dimensions of 3.5 × 20 mm, and made by Boston Scientific (Natick, Massachusetts, USA). A hybrid rosuvastatin/PLGA nanofiber-mounted stent was thus formed. All manufactured stents were placed in a vacuum oven at 40 °C for 72 h to evaporate the solvents.

2.2. Effect of rosuvastatin loading on platelet adhesion in vitro

Blood was drawn from a healthy rabbit and mixed with 3.2% sodium citrate in a volume ratio of 1/9 volume. Platelet-rich plasma (PRP) was obtained by centrifugation at 150G for 10 min. The blood was maintained at 22 °C before the PRP was separated out. The number of platelets per unit volume of PRP was 2×10^5 cells/μl, determined using a semi-automated hematology analyzer (SYSMEX F820). Fifty microliters of the platelet suspension which contained 10^7 platelets was then placed on the surfaces of nanofibers at two area densities (2 and 5 μg/mm^2), and incubated at 37 °C for three hours. Following incubation with PRP, the platelets were washed three times in phosphate-buffered solution (PBS). The platelets that adhered to fiber surface were mixed with 1% glutaraldehyde, and immersed in PBS, before being allowed to stand in the fixative for 60 min at 4 °C. The number of platelets that adhered to the nanofibers was determined by counting the adherent cells in the scanning electron microscopic (SEM) photographs. To count the cells on each surface, 20 rectangular fields were selected at random. The adherent cells in these fields in these photographs were counted manually. Based on the data thus obtained, the mean densities of the adherent platelets per square mm were calculated.

2.3. In vitro release of pharmaceuticals

The *in vitro* release characteristics of rosuvastatin from the nanofibers were determined using an elution method. Samples with a diameter of 3.5 mm and a length of 20 mm with two rosuvastatin loadings (2 and 5 μg/mm^2) were placed in glass test tubes (one sample per test tube, and three tubes for each loading) with 1 ml of PBS (0.15 mol/L, pH 7.4) in each test tube. Fresh PBS (1 ml) was added and the eluent was then allowed to stand for 24 h. The glass test tubes were then incubated at 37 °C for 24 h before the eluent was collected and analyzed. This procedure was repeated for 30 days.

The drug concentrations in the eluents were determined by performing an HPLC assay. The HPLC analyses were carried out using a Hitachi L-2200 multisolvent delivery system. An XBridge C_{18} 5 μm, 4.6 × 250 mm HPLC column (Waters) was used to separate out rosuvastatin. The mobile phase contained 99.9% acetonitrile (Mallinckrodt, U.S.A.) and ultra-pure water (40/60, v/v). The absorbency was monitored at a wavelength of 240 nm and the flow rate was 1.5 ml/min. All experiments were performed in triplicate and the sample dilutions were conducted to bring the unknown concentrations into the range of the standard curve of the assay. A calibration curve was plotted for each set of measurements (correlation coefficient > 0.99). The elution product was identified and quantified with high sensitivity using the HPLC system.

2.4. Surgical procedure and animal care

Adult male New Zealand white rabbits with a mean mass of 3.2 ± 0.2 kg were used in the animal study. The rabbits were housed in individual cages in a temperature- and light-controlled room and given standard rabbit chow *ad libitum* with free access to sterilized drinking water. All animal procedures were institutionally approved, and all of the animals were cared for in a manner consistent with the regulations of the National Institute of Health of Taiwan under the supervision of a licensed veterinarian.

Rabbits were sedated and anesthetized by a muscular injection of xylazine (9.3 mg/kg), and by administering Zoletil 50™ (tiletamine-zolazepam, 10 mg/kg) and oxygen (2 L/min) through a face mask. A 5F sheath was inserted into the femoral artery using the puncture technique. Hybrid stents on which were mounted nanofibers with two rosuvastatin loadings (2 and 5 μg/mm^2 for total doses of 500 and 1250 μg, respectively) were used *in vivo*. Twenty-four rabbits were separated into three groups. Group A consisted of eight rabbits, in which were deployed hybrid stents with a drug loading of 5 μg/mm^2; group B comprised eight rabbits that received hybrid stents with a drug loading of 2 μg/mm^2; group C comprised eight rabbits that received stents with virgin PLGA membranes (with no drug loading) as a control group. During the procedure, the rabbits firstly underwent endothelial denudation of the descending abdominal aorta using a 3.5 × 20 mm Maverick™ balloon (Boston Scientific, Maple Grove, Minnesota, USA) to cause an angioplasty balloon injury. The balloon was passed thrice over a 0.014″ guide wire to the aorta, inflated to nominal pressure (8 bars with 50% (v/v) contrast/saline) and withdrawn in a retrograde manner to the low descending abdominal aorta. Briefly, stents were deployed in the low descending abdominal aorta of each rabbit. When the stents had reached the target sites, they were expanded for 15 s (8 bars) to a diameter of 3.5 mm, yielding a ratio of stent-to-artery diameters of 1.2:1. Following stent implantation, post-procedural angiography was conducted to record vessel patency before the animals were allowed to recover. All rabbits were anti-coagulated with acetylsalicylic acid (40 mg/day) that was administered orally 24 h before catheterization and with a continuously dosage throughout the in-life phase of the investigation. A single dose of intra-arterial heparin (150 IU/kg) was administered upon catheterization.

2.5. In vivo evaluation of endothelial function

On day 28, endothelium-dependent vasomotor function at 5–10 mm distal from non-stented reference segments was evaluated following the infusion of two incremental doses of acetylcholine (Ach, 0.05 and 0.5 μg/ml/min) through the marginal ear vein. Ach was delivered via an infusion pump (Harvard Apparatus, Holliston, MA, USA) at 1 ml/min for three minutes, with intervals of precisely five minutes between each injection. Endothelium-independent function was evaluated using nitroglycerine (NTG, 5 μg/ml/min). Angiography was performed before and 30 s after each drug administration.