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Comparison of phytoncide with sirolimus as a novel drug candidate for drug-eluting stent

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ABSTRACT

A drug-eluting stent (DES) is one of the commonly used treatment techniques in percutaneous coronary intervention (PCI). Sirolimus (SRL) has been widely used for DES as a drug for suppressing neointimal hyperplasia causing restenosis. Phytoncides (PTC) are compounds released from trees and plants, and their solutions contain monoterpenoids such as α-pinene, careen, and myrceen. Some studies have reported that these components exhibit antioxidant, antimicrobial, and anti-inflammatory activities. We hypothesized that PTC may become an alternative drug to SRL for DES, exhibiting alleviated side effects as compared to SRL. A PTC-incorporated stent was compared with an SRL-incorporated stent in terms of physicochemical, pharmacokinetic, and biological properties. In *in vitro* studies, the effects of each drug on cells were investigated. The results showed that both drugs exhibited similar cytotoxicity, anti-inflammation, and antiproliferation effects. However, these effects resulted from different mechanisms associated with cells, as seen in the immunofluorescence result. An *in vivo* assay showed that the lumen area was significantly larger and the neointimal area was significantly smaller in SRL- and PTC-loaded stents compared to a drug-unloaded stent. These results suggest that phytoncide can be a feasible alternative drug to SRL for advanced DES although more studies are needed.

1. Introduction

A drug-eluting stent (DES) is one of the well-known treatment techniques in percutaneous transluminal coronary angioplasty (PTCA). A DES is a metallic stent coated with drug-incorporated non-degradable/degradable polymers. DES was developed to prevent the risk of restenosis that was commonly observed when using a bare metal stent (BMS) [1]. DES, which can release drugs such as sirolimus (SRL) and paclitaxel, has been successful because it significantly reduces the rate of restenosis [2–5]. Studies are increasingly focusing on DES and realizing improvements. One need that has emerged is to improve drugs for adequate efficacy without side effects.

SRL is known not only to reduce neointimal hyperplasia via the restrained proliferation and migration of vascular smooth muscle cells (VSMCs) but also to induce tissue factor (TF) that leads to thrombosis in human endothelial cells (ECs) by inhibiting the activity of the mammalian target of rapamycin (mTOR) [6–10].

Phytoncides (PTCs) are volatile substances released from trees and plants. They exhibit many effects, such as anti-oxidative effect, and have been shown to cause a decline in multiple sclerosis. Some studies have reported that terpenoids, a component of PTC solutions, exhibit antioxidant and antimicrobial activity [11–15]. A study showed that PTC has a protective effect on physical and chemical oxidative stress. Duka et al. reported that PTC is not toxic to humans and that it reduces the sympathetic activity under stress and blood pressure in humans and animals [14]. Chemical and pharmacological studies of PTC have shown that some of its

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components exhibit antigastropathic, anti-inflammatory, and antioxidant activity [11–15]. Most drugs used for DES are relatively lower molecular weight lipophilic compounds such as rapamycin, paclitaxel, or SRL analogs. In all existing DESs, drugs are homogeneously dispersed in the polymeric matrices. Therefore, the release kinetics is mainly governed by the diffusion mechanism of a drug through a coating matrix. These drugs are relatively larger than PTC, whose molecular weight ranges from 130 to 160 [15].

For controlled release, the drug for DES needs to be coated with a drug carrier matrix. Poly(lactic-co-glycolic acid) (PLGA) is widely known as a drug carrier, and it was therefore used in the present study [16,17]. This polymer has been shown to degrade mainly by the simple hydrolysis of the ester group to produce acidic monomers that can be removed from the body by normal metabolic pathways [18]. PLGA have been widely used in tissue engineering research because of its low immunogenicity, good biocompatibility, and excellent mechanical properties [16–18].

An ultrasonic nanocoating method was used for controlled drug release from the coated polymer [19]. This method is advantageous in that it is cost-effective and defect-free and achieves a uniform coating. It may provide beneficial surface properties such as enhanced biocompatibility, and this may enable prediction of the release mechanism in polymer matrix for the composition of a coating film that is regulated and flat [20].

In this study, PTC was, for the first time, compared with SRL to investigate its feasibility as an alternative drug for DES in terms of physicochemical, pharmacokinetic, and biological properties.

2. Materials and methods

2.1. Materials

A stainless steel (SS, 316L, KISTEC, Korea) plate (10 × 10 mm^2) and a cobalt–chromium (Co–Cr) stent (diameter: 1.8 mm, length: 18 mm, Bioalpha Inc., Korea) were used for the *in vitro* experiments and *in vivo* animal test, respectively. Poly(lactic-co-glycolic acid) (PLGA 75:25, M_W = 268,000 g/mol) was purchased from Boehringer Ingelheim (Ingelheim, Germany) for the film (10 × 10 mm^2) and the coating substrate, respectively. Acetonitrile, dioxane, chloroform, dichloromethane, and methanol were purchased from Aldrich (St. Louis, MO, USA). SRL was purchased from LC Laboratories (MA, USA). Phytoncide (PTC) extracted and refined from Cypress trees was donated by Jeonnam Nanobio Research Center (Korea). Fresh frozen plasma and human platelet concentrate were obtained from the Blood Center of the Korea Red Cross.

Human umbilical vein endothelial cell (HUVEC) and human coronary artery smooth muscle cell (SMC) were purchased from Lonza (Walkersville, USA). Endothelial basal medium-2, EGM-2 supplement kit (FBS: 10 mL, hydrocortisone: 0.2 mL, hFGF-B: 2 mL, VEGF: 0.5 mL, R3-IGF-1: 0.5 mL, ascorbic acid: 0.5 mL, hEGF: 0.5 mL, GA-1000: 0.5 mL, Heparin: 0.5 mL), smooth muscle basal medium, and SmGM supplement kit (insulin: 0.5 mL, hFGF-B: 1 mL, GA-1000: 0.5 mL, FBS: 25 mL, hEGF: 0.5 mL) were purchased from Lonza (Walkersville, USA).

2.2. Preparation of drug-loaded PLGA films and coating substrates

For preparation of drug-loaded PLGA films, PLGA was dissolved in chloroform (0.5% w/v) and added with the different amounts of SRL or PTC in the solution. The solution poured in a mold (10 × 10 cm^2) and dried for 5 days in air completely. In addition, to obtain 0.5% (w/w) polymer coating solution, PLGA was dissolved in a co-solvent of dioxane and dichloromethane (50:50), and different amounts of SRL or PTC were added to the solution. Each percentage (10, 30, and 50 wt%) of drug was mixed with the PLGA solution and coated on the stainless steel and Co–Cr substrates using an ultrasonic nanocoater (SONO-TEK, USA). An ultrasonic nanocoating method was carried out following coating conditions; flow rate of 0.05 mL/min, eluting amount of PLGA solution of 0.5 mL, and rotating velocity of mandrel of 100 rev./min. The coating was performed for 20 min by separately coating 2 times at a relative humidity of 40–50% and room temperature. The coated substrates were air-dried for 1 day and maintained under vacuum for 1 day to remove the residual solvent.

2.3. Characterizations of drug-loaded PLGA coating substrate

The wettability of drug-loaded PLGA coating substrate with three different amounts of drug was measured using a water contact angle analyzer (DGD Fast/60 Contact Angle Meter, GBX New Technologic Development, France). The substrate was placed on the testing plate. Distilled water was carefully dropped on the substrate surface. Bare stainless steel was used as a control.

The molecular weight of PLGA coated on the substrate was determined using gel permeation chromatography (GPC, Waters, USA). Chloroform was used as a mobile phase with a flow rate of 1 mL/min. The coating layer was dissolved in 1 mL of chloroform and then filtered through a syringe filter (0.2 μm, Whatman). The injection volume of each vial was 200 μL. The data collection and analysis were performed using the Waters Millennium software. The weight average molecular mass was calculated using PLGA standards.

X-Ray diffraction (XRD) analysis was performed with an in-plane high-resolution X-ray diffractometer (HR XRD, Rigaku, Japan) equipped with CuKα radiation. Pure SRL and PTC were analyzed in the range of 2–40° with an angular resolution of 0.02°. Drugs were prepared on the quartz holder (Gem Dugout, PA, USA) having a low X-ray background.

For differential scanning calorimeter (DSC) analysis, PLGA only or drug-loaded PLGA films were prepared. The thermal property of PLGA films with drug was carried out by DSC (DSC Autosampler A2910900, TA Instrument, USA) in the second heating run. About 8.2 mg of films was packed in an aluminum pan and then heated from 0 to 250 °C at a ramp rate of 10 °C/min with nitrogen purge of 20 mL/min. The melting temperature (T_m) of each film was measured in the first run, and the glass transition temperature (T_g) was obtained in the second heating run.

The coating thickness and morphology of each sample were measured by field-emission scanning electron microscopy (FE-SEM, S-4100, Hitachi, Japan). The surface of the drug-loaded PLGA-coated substrate was sputtered with Pt under Ar atmosphere for 80 s before observation.

2.4. In vitro drug release test

The amounts of SRL and PTC in the PLGA matrix were determined using a UV spectrophotometer (λ = 286 nm) and high-performance liquid chromatograph (HPLC, LC 1100, Agilent, Germany), respectively. For an *in vitro* drug release test, samples were placed in a 10 mL vial with 2 mL of phosphate-buffered saline (PBS, pH 7.4) solution at 37 °C for various times. HPLC was measured using a mobile phase composed of acetonitrile, methanol, and water (45:40:15) at a flow rate of 1.2 mL/min and detection of 278 nm.

2.5. Protein adsorption test

For the fibrinogen adsorption test, PLGA or drug-loaded PLGA-coated substrates were hydrated with 2 mL PBS solution for 30 min at 37 °C and then immersed in Alexa Fluor 488 conjugated fibrinogen solution (0.2 mg/mL) (Invitrogen, USA) for 2 h at 37 °C. The samples were washed with distilled water, and then, fibrinogen adsorbed on each sample was detected by fluorescence microscopy (CKX41, Olympus, Japan). The fibrinogen amount on each sample was compared by the fluorescence intensity calculated using Image J software.

2.6. Platelet adhesion test

PLGA or drug-loaded PLGA-coated substrates were hydrated in 2 mL PBS solution at 37 °C for 1 h. The samples were immediately placed in the platelet-rich plasma (PRP) solution (1 × 10^5 platelets/μL) with platelet-poor plasma (PPP) solution and then transferred to a 12-well plate, following which incubation was conducted at 37 °C for 2 h. The samples were washed and fixed with 2% glutaraldehyde in PBS solution. The platelet-adhered samples were carefully washed with 50, 70, 90, and 100% ethanol solution sequentially for platelet dehydration and dried in vacuum. Platelets adhered on the samples were observed by FE-SEM. The number of platelets adhered on the samples was determined by lactate dehydrogenase activity (LDH) assay kit (Takara Bio Inc., CA, USA).

2.7. Cytotoxicity and cell proliferation assay

HUVEC and SMC (1 × 10^4 cells/well) were seeded in 24-well plates and incubated for 24 h and then treated with various concentrations of SRL or PTC for 72 h. For cell proliferation assay, cells (1 × 10^4 cells/well) were seeded into 24-well plates. Cells were incubated for 1 day and then treated with different concentrations of PTC for 1, 3, and 5 days. Cytotoxicity was analyzed using cell counting kit-8 (CCK-8) (Dojindo Molecular Technology, Inc., Rockville, USA).

2.8. Inflammation test

U937 cells (ATCC, USA) were differentiated by phorbol 12-myristate 13-acetate (PMA, Sigma, USA) for 48 h. Differentiated U937 cells (5 × 10^4 cells/well) or SMC (5 × 10^3 cells/well) were seeded in 24-well plates and treated with lipopolysaccharide (LPS, Sigma, USA) and then treated with various concentrations of SRL or PTC for 24 h. Supernatant was collected and analyzed by tumor necrosis factor-α (TNF-α) ELISA kit (R&D systems, USA) to U937 and interleukin-6 (IL-6) ELISA kit (R&D systems, USA) to SMC, respectively. In addition, cell viability was confirmed by CCK-8.

2.9. Immunofluorescence staining

SMC (5 × 10^4 cells/mL) were seeded on PLGA or drug-loaded PLGA-coated substrates and incubated at 37 °C for 5 days. Cells were fixed with formaldehyde, and then, F-actin and nuclei were stained with phallotoxins (Invitrogen, USA) and DAPI (Thermo, USA), respectively. Phallotoxins and DAPI stainings were performed by