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Polymeric stent materials dysregulate macrophage and endothelial cell functions: Implications for coronary artery stent

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ABSTRACT

Background: Biodegradable polymers have been applied as bulk or coating materials for coronary artery stents. The degradation of polymers, however, could induce endothelial dysfunction and aggravate neointimal formation. Here we use polymeric microparticles to simulate and demonstrate the effects of degraded stent materials on phagocytic activity, cell death and dysfunction of macrophages and endothelial cells.
Methods: Microparticles made of low molecular weight polyesters were incubated with human macrophages and coronary artery endothelial cells (ECs). Microparticle-induced phagocytosis, cytotoxicity, apoptosis, cytokine release and surface marker expression were determined by immunostaining or ELISA. Elastase expression was analyzed by ELISA and the elastase-mediated polymer degradation was assessed by mass spectrometry.
Results: We demonstrated that poly(D,L-lactic acid) (PLLA) and polycaprolactone (PCL) microparticles induced cytotoxicity in macrophages and ECs, partially through cell apoptosis. The particle treatment alleviated EC phagocytosis, as opposed to macrophages, but enhanced the expression of vascular cell adhesion molecule (VCAM)-1 along with decreased nitric oxide production, indicating that ECs were activated and lost their capacity to maintain homeostasis. The activation of both cell types induced the release of elastase or elastase-like protease, which further accelerated polymer degradation.
Conclusions: This study revealed that low molecule weight PLLA and PCL microparticles increased cytotoxicity and dysregulated endothelial cell function, which in turn enhanced elastase release and polymer degradation. These indicate that polymer or polymer-coated stents impose a risk of endothelial dysfunction after deployment which can potentially lead to delayed endothelialization, neointimal hyperplasia and late thrombosis.

1. Introduction

Drug-eluting stents (DESs) have remarkably reduced acute in-stent thrombosis and the occurrence of revascularization. However, late stent thrombosis associated with DES still remains unexpectedly high, which can be partially attributed to endothelial dysfunction and vasomotor disorders caused by stent, drug or polymer coatings [1]. Besides the stent geometry, the influence of incorporated drugs on inducing endothelial dysfunction has been studied in both animals and humans over the past decade [2–4]. In contrast, polymer coating-induced endothelial dysfunction has yet been reported despite their proven hypersensitive effect. Biodegradable and resorbable polymers provide appealing substitutes to durable polymer coatings, since they are less likely to induce severe chronic inflammatory response and their degradation products can be cleared out of the human body. Coronary artery stents coated with or completely made of biodegradable polyesters, i.e. poly(lactic acid) (PLA), poly(lactic-*co*-glycolic acid) (PLGA) and to a lesser degree, poly(ε-caprolactone) (PCL), have been developed [5–9]. These polymers undergo hydrolysis and enzyme-mediated degradation ("biodegradation") [10,11], resulting in a release of low molecular weight degradation products. The released polymer debris is then removed by leukocytes and foreign body giant cells ("bioresoprtion"), as indicated by enhanced phagocytosis and cell activation. Activated leukocytes subsequently alter the phenotype of surrounding cells (i.e. endothelial cells) and make them actively involved in clearing and remodeling processes. Endothelial cells (ECs) are normally quiescent and maintain vascular homeostasis. The activation of endothelium promotes the adhesion of platelets and leukocytes while losing its cell–cell integrity, which gradually leads to vascular malfunctions such as atherosclerosis and stenosis [12]. For example, neointimal formation

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has been found with the degradation of polymer stents [13,14], as revealed by locally elevated inflammation and cell growth to intima. Although it can be alleviated by timed-release anti-proliferative drugs, locally delivered therapeutics often induce EC apoptosis/necrosis, impair re-endothelialization and lead to late thrombosis [15,16]. It is unclear, however, whether there is a casual relationship between polymer debris and endothelial activation/dysfunction. Therefore, the objective of this study is to understand the interaction between polymer debris and ECs, which is particularly meaningful for polymer coronary stents. Here we use low molecular weight poly(D,L-lactic acid) (PLLA) and PCL microparticles as model molecules of degradation products to facilitate cellular uptake and to elucidate how macrophage and arterial endothelial cell functions can be regulated by degraded polymers. The results of this study will help interpret the occurring restenosis and late-thrombosis in polymer-coated stents and warrant more careful evaluation of endovascular polymer devices in general. It will enlighten both clinicians and biomedical engineers to improve the design of polymer-involved coronary stents from a perspective of minimizing the deteriorating effect of polymers on the endothelium.

2. Methods

2.1. Preparation of stainless steel, PLLA and PCL microparticles

PLLA (RESOMER R202S, 10–18 k Da, Sigma, St. Louis, MO) or PCL (1.25 k Da, Sigma) was dissolved in dichloromethane (10% w/w) and the polymer solution was added to 1% poly(vinyl acid) (PVA) water solution drop-wise under continuous stirring at 3000 rpm (Silverson L4RT homogenizer, East Longmeadow, MA). The polymer solution was then slowly stirred at room temperature overnight to evaporate the organic solvent. The PVA was removed by repeated washing with DI water and the particles were collected by centrifugation and lyophilizing overnight. Particles less than 38 μm were obtained by sieving (H&C Sieving Systems, Columbia, MD). Stainless steel (SS) particles with around 10 μm in diameter were prepared by grinding and served as controls throughout this study. Particle size and morphology were visualized using a Hitachi S4200 high resolution scanning electron microscope (SEM, Schaumburg, IL) (Fig. 1).

2.2. Cell culture

Human blood-derived monocytes were obtained from Advanced Biotechnologies (Columbia, MD) and differentiated into human blood monocyte-derived macrophages [17]. Monocytes at a density of 2×10^6 cells/mL were incubated with 10 mL DMEM (Invitrogen, Carlsbad, CA) with 20% fetal calf serum (Invitrogen), 10% human serum (Sigma), and 5 ng/mL macrophage colony-stimulating factor (Sigma) for 9 days to differentiate into macrophages [18]. Human coronary artery ECs (HCAECs, Cell Applications, San Diego, CA) were cultured in endothelial cell growth medium (Cell Applications) until 80% confluent before seeding. Polymer or SS particles were UV sterilized for 1 h prior to cell seeding. Macrophages (3×10^5 cells/mL) or HCAECs were cultured with polymer or SS particles (400 μg/mL) for 3 days before endpoint assays.

2.3. Phagocytosis and superoxide assays

After 3 days of culture, macrophages or HCAECs were treated with green-fluorescent *Escherichia coli* (*E. coli*) particles for 2 h according to the manufacturer's protocol (Vybrant® Phagocytosis assay kit, Life Technologies, Carlsbad, CA). Cells were stained with dihydroethidium (1 μg/mL DHE, Life Technologies) for 15 min. Cell nuclei were counterstained with Hoechst and imaged with an inverted Nikon Ti fluorescent microscope (Nikon Instruments, Melville, NY). Fluorescence intensities of *E. coli* particles phagocytized by activated cells and superoxide as indicated by DHE were measured using a plate reader (Tecan Group, Ltd. Männedorf, Switzerland). Phagocytic activity and superoxide production were normalized to cell number quantified by Hoechst nuclear staining (n = 4 per polymer condition).

2.4. Cytotoxicity

Macrophage and endothelial cell cytotoxicities were measured using CytoTox One homogeneous membrane assay (Promega, Madison, WI) by quantifying lactate dehydrogenase released from damaged cells according to supplier's protocol. Fluorescence values were normalized to cell samples without material treatment (n = 4 per polymer condition).

2.5. TNF-α and nitric oxide expression

After 3 days of culture, media samples were collected and analyzed for secretion of tumor necrosis factor (TNF)-α, elastase, and nitric oxide (NO). TNF-α secretion was measured using human TNF-α ELISA max standard kit (BioLegend, San Diego, CA) according to supplier's protocol. NO secretion was measured by incubating 100 μL 1× modified Griess reagent (Sigma) with 100 μL media or nitrate standards for 15 min before reading the absorbance at 540 nm using a plate reader (Tecan). Nitrate standards (0–65 μmol) were made using sodium nitrate (Sigma) (n = 4 per polymer condition).

2.6. Immunostaining of VCAM-1 and annexin V

Cells were fixed with 4% paraformaldehyde (PFA) for 15 min, blocked with 10% goat serum in Dulbecco's phosphate-buffered saline (DPBS) for 1 h, and incubated with allophycocyanin-conjugated VCAM-1 antibodies (1:100 dilution, Abcam, Cambridge, MA) for 2 h in DPBS. For annexin-V staining, HCAECs were fixed with 4% PFA for 15 min, permeabilized with 0.25% TritonX-100 for 10 min, blocked with 10% goat serum for 1 h, incubated with rabbit anti-human annexin V antibodies (1:200 dilution, Abcam) overnight at 4 °C, and then incubated with Alexa 488 goat anti-rabbit antibodies (1:00 dilution, Abcam) for 1 h in DPBS. After washing twice with DPBS, cells were imaged on a LSM 710 META inverted confocal microscope (Zeiss, Thornwood, NY). The number of cells that were positive to each staining was counted and divided by the total number of cells per image field (n = 6 images from three replicate experiments).

2.7. Elastase activity

In order to measure elastase activity, elastase standards (0–10 nmol) were made using human neutrophil elastase (Sigma). 200 μL of media samples or standards were incubated with 200 μL of 1 mM granulocyte elastase substrate (Sigma) in DPBS for 1 h at 37 °C and glacial acetic acid was added to stop the reaction. Absorbance was measured at 415 nm by a plate reader (Tecan) (n = 4 per polymer condition) [19].

2.8. Polymer degradation by elastase

PLLA particles were suspended in DPBS with or without elastase (0.08 U/mL, Sigma), and incubated at 37 °C for 7 days. Insoluble polymer particles were filtered off using centrifugation units (Amicon-0.5 mL 3 K MWCO, Millipore, Billerica, MA) at 12,000 *g* for 20 min. Degradation products in the filtered solution were confirmed by liquid chromatography coupled with mass spectrometry (LC–MS) (Variant 1000, Agilent, Santa Clara, CA) using a C18 column (Kinetex, Phenomenex, Torrance, CA) with an acetonitrile–H_2O mobile phase system (n = 3 per polymer condition).

2.9. Statistical analysis

In all experiments, analytical results are expressed as means ± standard error of the mean (SEM). One-way ANOVA was used to determine if statistical differences exist between groups. Comparisons of individual sample groups were performed using unpaired Student's *t*-test. For all experiments, $p < 0.05$ was considered statistically significant.

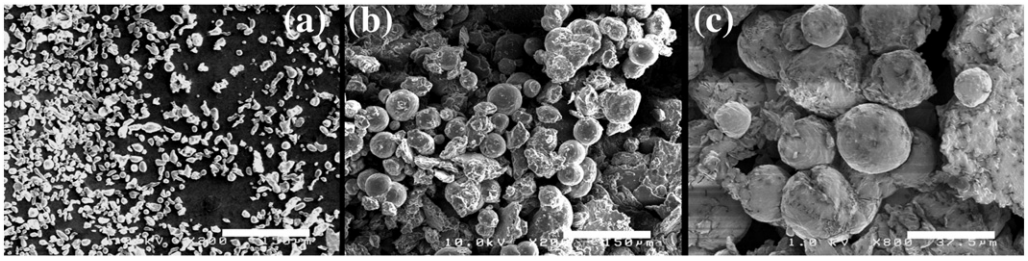

Fig. 1. SEM images of microparticles: (a) stainless steel, (b) PLLA and (c) PCL. Scale bar: 150 (a and b) or 37.5 (c) μm.