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Enhanced Endothelialization of a New Stent Polymer Through Surface Enhancement and Incorporation of Growth Factor-Delivering Microparticles

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Abstract In this study, we sought to develop strategies for improved endothelialization of a new polymer coating for vascular stents. Surface enhancement of the new poly-1,8-octanediol-co-citric acid (POC) polymer was achieved through conjugation of anti-CD34 antibody and incorporation of vascular endothelial growth factor and basic fibroblast growth factor-containing poly-lactic-co-glycolic acid microparticles to improve capture and proliferation of endothelial progenitor cells (EPC) and compared to untreated POC and poly-L-lactic acid (PLLA) polymer. Our results indicate that compared to PLLA, POC coating was more hemocompatible, with less platelet activation ($p=0.01$), thrombogenicity ($p<0.05$ for 20 and 30 min clot formation), and inflammatory response (IL-1β release, $p=0.0009$; TNF-α release, $p=0.004$). EPC adhesion and proliferation on POC were significantly improved with surface enhancement and microparticle incorporation compared to untreated POC ($p=0.006$) and PLLA ($p=0.003$). These results suggest a new strategy for enhancing endothelialization of polymeric coatings of vascular prostheses.

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Introduction

Percutaneous transluminal coronary intervention, particularly with stent implants, has become the preferred revascularization treatment for obstructive coronary artery diseases due to the significant reduction in restenosis compared to balloon angioplasty. However, evidence suggests that metal stents with antiproliferative drugs [drug-eluting stents (DES)] loaded on polymers covering the stent strut may be at an increased risk of late stent thrombosis (LST), a catastrophic event with significant morbidity and mortality rate [1].

Although the mechanism of LST is likely multifactorial and is not completely understood, recent studies have suggested that delayed arterial healing of the stent polymer may play an important role [2, 3]. Formation of an intact and functional vascular endothelial cell layer on the polymer surface plays a pivotal role in inhibiting platelet aggregation and clot formation [4]. The deployment of DES, resulting in endothelial damage and delayed endothelial progenitor cell (EPC) layer formation, may impose a greater risk for LST [4, 5]. Thus, an expedited arterial healing strategy with enhanced endothelialization of the stent polymer might reduce the incidence of LST.

In this study, we report strategies to enhance endothelialization of a new stent polymer POC (poly-1, 8-octanediol-co-citric acid) using surface enhancement with anti-CD34 antibodies (Ab) immobilized on its surface and embedded with growth factor-delivering polymeric microparticles and compare it to a widely used and well-accepted biocompatible polymer, PLLA (poly-L-lactic acid).

Materials and Methods

Preparation of Modified POC Coating

POC, an elastic polymer, was synthesized as described previously [6]. Poly-lactic-co-glycolic acid (PLGA) was used to construct growth factor-delivering microparticles by encapsulating vascular endothelial growth factor (VEGF) and beta-fibroblast growth factor (bFGF) within the particle matrix using a double-emulsion method [7]. In this study, we loaded 5 μg VEGF or bFGF in 250-mg PLGA microparticles. Scanning electron microscopy (SEM; Hitachi S-3000N) was used to examine particle morphology. To make the POC coating, 1.5 g POC pre-polymer, 17 mg PLGA microparticles encapsulating VEGF, and 17 mg PLGA microparticles encapsulating bFGF were mixed and spread evenly on glass cover slips then polymerized in a freeze drier. Human fibronectin followed by mouse anti-human CD34 antibody (both 33 μg/ml, Abcam Inc.) were conjugated on the polymer surface using covalent coupling method [8].

Preparation of Modified PLLA Coating

PLLA was dissolved in chloroform to make a 2.5 % (*w*/*v*) solution then casted on glass cover slips to form films when dried [9]. The films were washed with ethanol, deionized water, and sterilized under ultraviolet light for 30 min. Surface enhancement of PLLA with anti-CD34 Ab was performed as described above with POC. Incorporation of PLGA microparticles within PLLA is not feasible, given their similar solubility in chloroform and other commonly used solvents [10].

Characterization of Growth Factor Release Kinetics

To assess growth factor release kinetics, 20 mg PLGA microparticles was suspended in 1.5 ml phosphate-buffered saline and incubated with gentle shaking at 37°C. At predetermined time points, VEGF and bFGF levels were measured using enzyme immunoassay kits (Invitrogen Inc.).

Hemocompatibility of POC Coating

Platelet adhesion on POC coating was examined by detecting the amount of lactate dehydrogenase when the adhered cells were lysed after an hour of incubation [11]. Platelet activation was examined by flow cytometric analysis of CD62P (platelet membrane-bound P-selectin) positive events among all the platelets [12]. Kinetics of whole blood clotting was measured as described by Huang et al. to evaluate resistance to thrombus formation [13]. Inflammatory response of cells exposed to POC coating was evaluated by measuring release of interleukin-1beta (IL-1β) and tumor necrosis factor alpha (TNF-α) using cytometric bead array (BD Inc.). Red blood cell hemolysis was also examined [14]. PLLA was also treated with fibronectin and anti-CD34 Ab and compared to POC.

EPC Culture and Characterization

The ethics committee of the University of Texas at Arlington approved the study, and each participant provided a written informed consent. Endothelial progenitor cells (EPC) were isolated from the peripheral blood of healthy volunteers using density gradient centrifugation with Ficoll-Paque (GE Healthcare, Sweden) and cultured with EGM-2 medium [15]. To characterize EPC, we examined several EPC functional and phenotypic markers including EPC colony morphology, uptake of Dil-acetylated low-density lipoprotein (Dil-Ac-LDL), immunofluorescence staining for AC133, CD34, and von Willebrand Factor (vWF) [16].

EPC Capture Under Flow Conditions and EPC Proliferation

An in vitro parallel flow system was used to study EPC capture under shear stress [9, 17]. Briefly, POC or PLLA films were coated on glass slides, treated with fibronectin and anti-CD34 Ab, and then aseptically mounted on flow chambers. Untreated polymers served as controls. EPC suspension (4×10^6 cells) was allowed to flow over the polymer films at 3.5 ml/min. After 24 and 48 h, arrested EPC on the polymer films were observed using an inverted microscope (Zeiss Axiovert CFL). The number of cells at five randomly picked areas was counted.

To study EPC proliferation, the cells were seeded at 2,000 cells/cm^2 on POC coated with anti-CD34 Ab, and the experiment was repeated with incorporation of growth factor-delivering microparticles. Again, POC without anti-CD34 Ab served as control. POC (+/− anti-CD34 Ab) was then compared to PLLA with and without anti-CD34 Ab conjugation. EPC proliferation at days 3 and 7 were examined using PicoGreen DNA assay (Invitrogen Inc.).

Data Analysis

All data are presented as mean±standard deviation. Statistical analysis was unblinded; one-way ANOVA was used to compare the differences between groups, and $p<0.05$ was deemed as statistically significant.

Results

Synthesis of Growth Factor-Delivering Microparticles

As shown in Fig. 1a, synthesized PLGA microparticles are spherical with smooth surfaces. The diameter of these particles is between 50 and 100 μm. The cross-sectional view clearly demonstrates the multiple pores inside the microparticle matrix (Fig. 1b). The loading efficiency of VEGF or bFGF in the PLGA microparticles was calculated to be around 68 and 61 %, respectively. Encapsulated VEGF and bFGF were released over an 8-week period (Fig. 1c). During this period, 121 ng of VEGF and 74 ng of bFGF were released, accounting for 33.5 % of loaded VEGF and 20 % of loaded bFGF.

Hemocompatibility of POC Coating

As shown in Fig. 2, percent platelet activation to POC was significantly less than that to PLLA (0.17 vs. 0.75 %, p=0.01). Platelet adhesion was similar on POC and PLLA. Clot formation was significantly slower on POC and PLLA compared to glass (positive control) after exposure of whole blood, recalcified with the addition of calcium chloride (final concentration 0.01 M) for 20 min (p=0.02, POC vs. glass; p=0.02, PLLA vs. glass) and 30 min (p=0.005, POC vs. glass; p=0.007, PLLA vs. glass), respectively. A stable clot was formed on all substrates at 60 min. IL-1β release, when anticoagulated whole blood (with ethylenediaminetetraacetic acid) was exposed to POC for 60 min, was 6.625 pg/ml, significantly less than when exposed to PLLA (11.79 pg/ml, p=0.0009). TNF-α release was also significantly less with POC than PLLA (5.96 vs. 10.3 pg/ml, p=0.004). RBC hemolysis on POC and PLLA was 0.5 and 0.27 %, respectively (p=0.17).

EPC Characterization

EPC presented elongated fibroblast-like morphology (Fig. 3a) and formed typical colony-forming units, characterized by a central core of round cells with spindle-shaped cells around the periphery (Fig. 3b) during the first week of cell seeding. There was uptake of Dil-Ac-LDL (Fig. 3c), and the cells were positively stained with AC133, CD34, and vWF (Fig. 3d–f), characteristic of EPC.

EPC Capture and Proliferation on POC

As shown in Fig. 4, after 2 days of seeding under consistent unidirectional flow, adhered EPC on surface-enhanced POC (2,700 cells/mm^2) were significantly greater than that on surface-enhanced PLLA (1,150 cells/mm^2, p=0.02). The

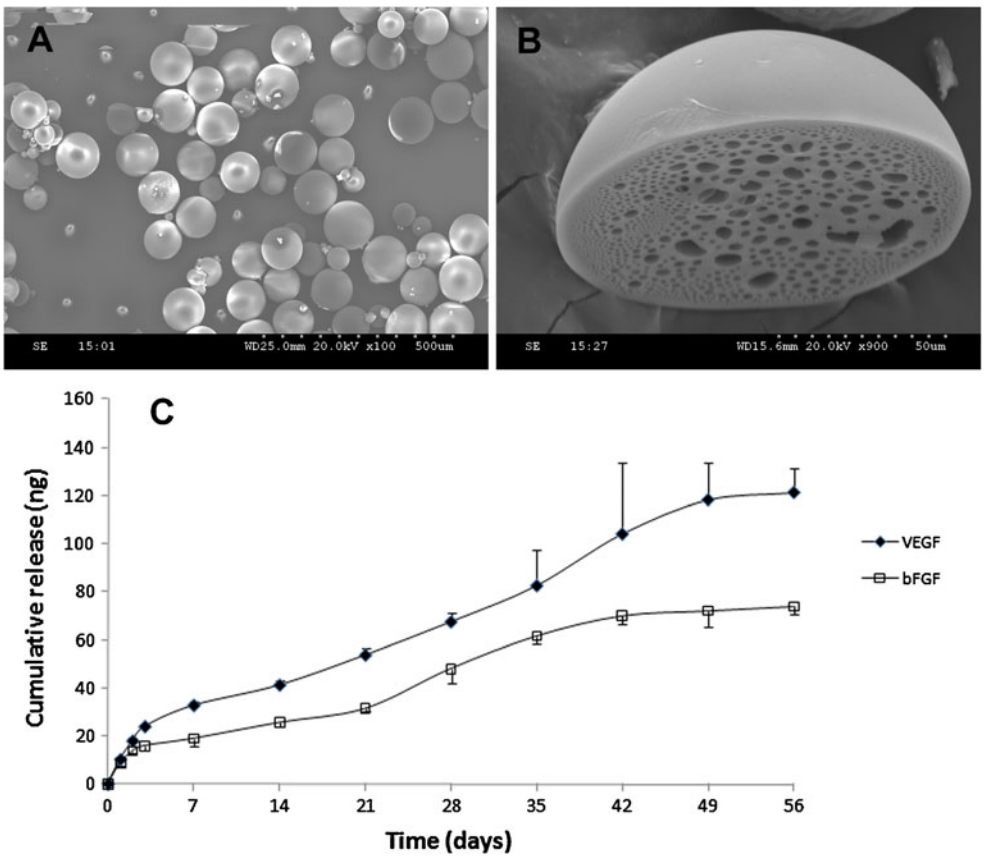

Fig. 1 PLGA microparticles. **a**–**b** Representative SEM images of PLGA microparticles encapsulating VEGF or bFGF. **c** Cumulative release of growth factors (VEGF and bFGF) from PLGA microparticles in vitro. Data are presented as mean± SEM (n=4)

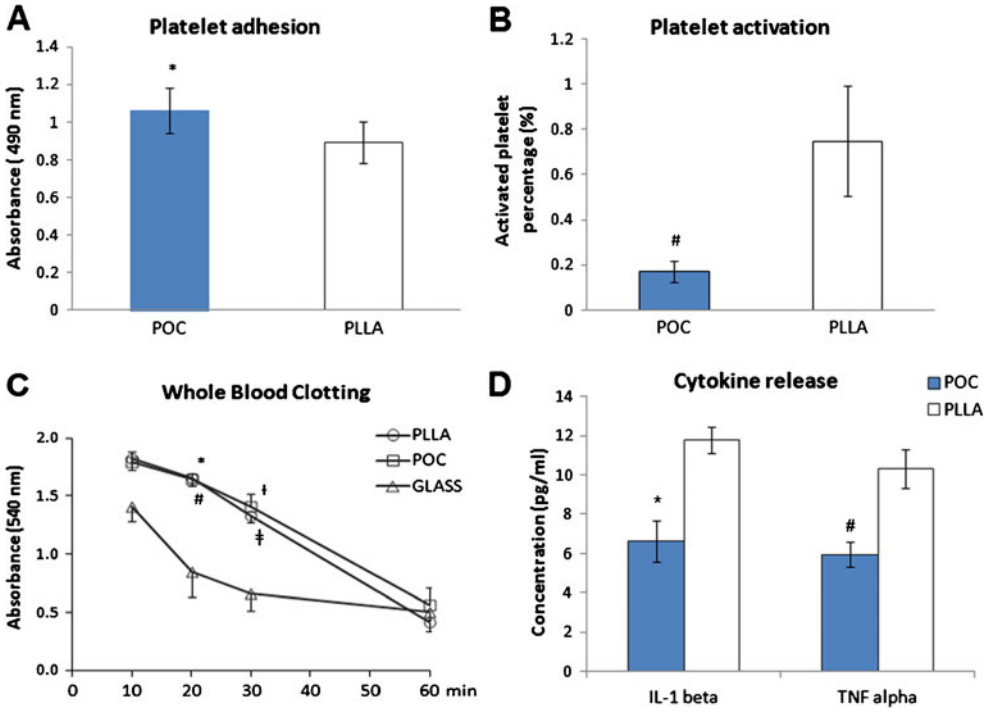

Fig. 2 Hemocompatibility assessment of the POC coating. **a** Platelet adhesion was assessed by measuring lactate dehydrogenase of lysed platelet adhered on the polymer surface after a 1-h incubation. $n=4$, $^{*}p=0.33$ for POC vs. PLLA. **b** Platelet activation was examined by flow cytometric analysis of CD62P-positive events among all the platelets after a 1-h incubation with the polymers. $n=9$, $^{\#}p=0.11$ for POC vs. PLLA. **c** Whole blood clotting was determined by measuring the free hemoglobin released from lysed red blood cells that were not trapped in blood clots at different time points when whole blood was incubated on the polymer surfaces. $n=4$. $^{*}p=0.02$ for POC vs. glass, $^{\#}p=0.02$ for PLLA vs. glass; $p=0.005$ for POC vs. glass; $p=0.007$ for PLLA vs. glass. **d** IL1-β and TNF-α release was measured after a 1-h blood incubation on the polymer surfaces. IL1-β: $n=6$, $^{*}p=0.008$ for POC vs. PLLA; TNF-α: $n=6$, $^{\#}p=0.004$ for POC vs. PLLA. All data are presented as mean±SEM

cells arrested on the polymer coatings presented a stretched morphology, with their longitudinal axis parallel to the direction of flow. No cells were found adhered on untreated (anti-CD34 Ab) POC or PLLA.

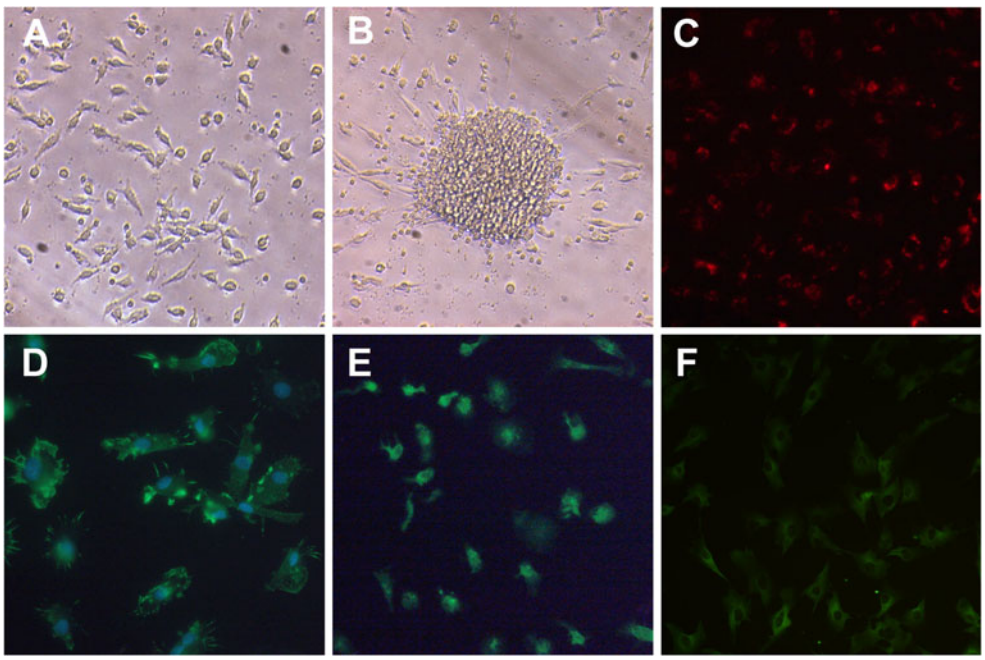

Fig. 3 EPC culture in vitro. **a** Phase contrast image of EPC cultured on fibronectin-coated tissue culture flasks for 3 days. **b** EPC colony-forming unit. **c**–**f** Fluorescent images of EPC uptake Dil-ac-LDL (**c**), or stained with AC133 (**d**), CD34 (**e**), or vWF (**f**)

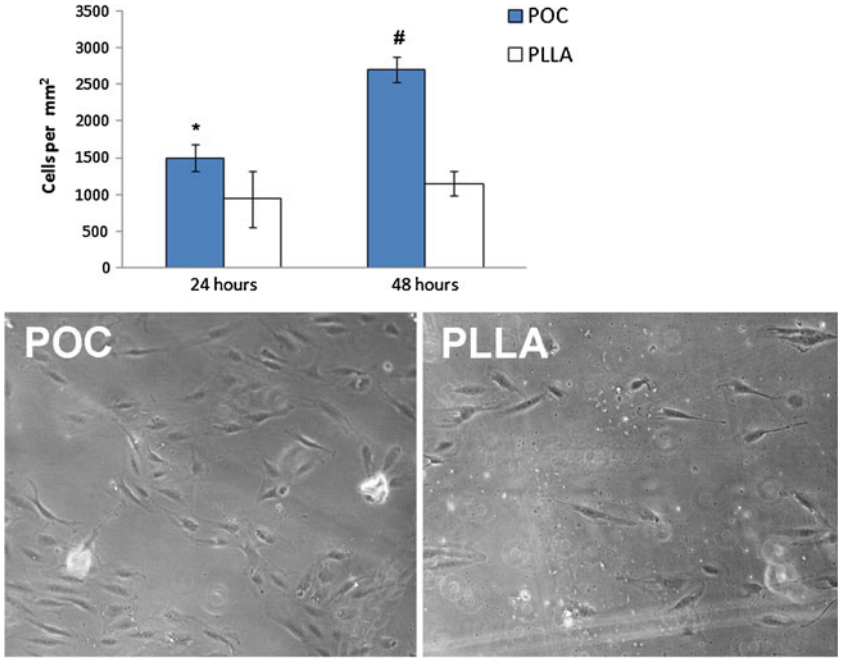

Fig. 4 EPC adhesion on POC coating under flow condition. (*Top*) EPC adhered on surface enhanced POC or PLLA under continuous flow (3.5 ml/min) for 48 h. The number of cells at five randomly picked areas was counted. Data are presented as mean±SEM. *p=0.06 for POC vs. PLLA at 24 h; $^{\#}p$=0.02 for POC vs. PLLA at 48 h. (*Bottom*) representative images of EPC adhered on surface-enhanced POC or PLLA after a 48-h flow

As shown in Fig. 5, EPC proliferation (indicated by the amount of cell DNA) was significantly higher on POC when anti-CD34 Ab were conjugated on the surface compared to unmodified (anti-CD34 Ab) POC (50.5 vs. 35.3 ng/ml, p=0.04). The incorporation of microparticles alone did not increase EPC growth after 3 days of culture.

Rapid EPC proliferation was observed on the POC between days 3 and 7. At day 7, cells grown on microparticle-embedded POC showed significantly higher EPC proliferation than on untreated POC (160.5 vs. 80.3 ng/ml, p=0.0004). The highest cell proliferation was found on modified POC with both surface enhancement with anti-CD34 Ab conjugation and growth factor containing microparticle incorporation (197.9 ng/ml, p=0.006 compared to unmodified POC at day 7). Cell proliferation on untreated PLLA was 18.4 and 24.7 ng/ml at days 3 and 7, respectively, significantly less than that on POC (p=0.007 at day 3; p=0.002 at day 7). Surface enhancement of PLLA with anti-CD34 Ab led to minimal improvement in cell proliferation compared to unenhanced PLLA, both at day 3 (25.5 vs. 18.4 ng/ml, p=0.15) and day 7 (35.2 vs. 24.7 ng/ml, p=0.19).

Discussion

The findings of this study indicate that POC polymer modified with surface-conjugated anti-CD34 Ab and incorporated with VEGF/bFGF delivering-microparticles had superior hemocompatibility, supported better EPC capture and proliferation compared to PLLA.

Current polymer-coated stents are associated with delayed endothelialization, which is considered to be a significant risk factor for LST. Endothelial coverage is essential for maintaining long-term vessel patency after stenting as endothelial cells play a crucial role in preventing thrombosis. Based on correlative swine and rabbit studies [18, 19], it is estimated that complete endothelial coverage of bare metal stents in humans requires approximately 12 months, with even longer time periods needed for coverage of DES [5, 20]. While antiplatelet therapy is normally used for up to 1 year after stent implantation, uncovered stent struts may continue to pose a thrombogenic risk. As prolonged antiplatelet therapy is associated with increased risk of bleeding, a strategy of expedited endothelialization of stent polymers may provide a complementary and lasting solution.

After the initial discovery of EPC by Asahara et al. [21], these cells have been widely accepted as main contributors towards repairing the damaged endothelium after an endovascular injury [22, 23]. To utilize EPC for regeneration of endothelial cells on vascular prostheses, two main methods, "in vitro seeding" and "in vivo capture," have been traditionally employed. However, early studies show that EPC preseeded on stents are only partially retained after stent delivery, primarily related to an inevitable cell loss during the implantation procedure [24]. "In vivo capture," also called "auto-seeding," on the other hand is an innovative strategy to overcome these limitations. EPC-capturing biomolecules including anti-CD34 Ab [25], anti-VEGFR2 Ab [26], cRGD peptide [27], laminin-derived peptides sequence [28], phage display-selected peptide [29], or DNA aptamers [30] have all been tested to capture EPC

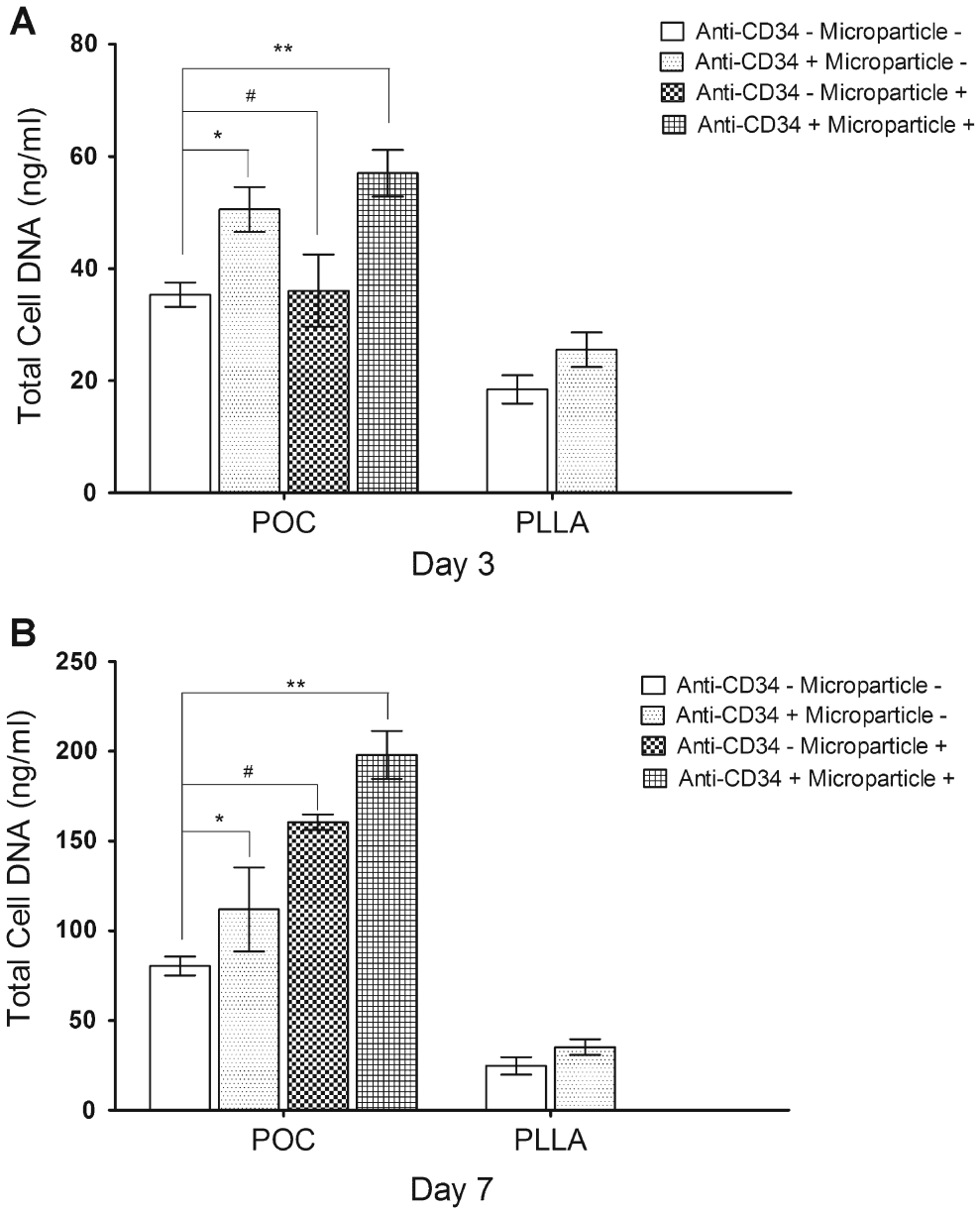

Fig. 5 EPC proliferation on POC coating: EPC proliferation on POC coating with or without anti-CD34 Ab conjugation, and with or without microparticle incorporation after 3 days (**a**) or 7 days (**b**) incubation. **a** N=4. Data are presented as mean± SEM. *p=0.04, $^{\#}p$=0.93, **p=0.02; **b** N=4. Data are presented as mean±SEM. *p=0.03, $^{\#}p$=0.0004, **p=0.006

on vascular prostheses. Recent animal studies and clinical trials have found that EPC capture on vascular prostheses improves endothelialization of stents and are safe for human use [25, 27, 31].

EPC capture technology alone may be limited by the number of circulating EPC in peripheral circulation and/or its mobilization following an endovascular injury, both of which can be highly variable [23]. Incomplete endothelial coverage is still found on EPC-capturing bioengineered R stent (Orbus Neich's Genous™) at 28 days following implantation in porcine coronary [32]. To accelerate endothelialization, additional growth factors may be needed to stimulate proliferation. It is well known that VEGF and bFGF are two such growth factors [33, 34]. In fact, VEGF and bFGF are both included in the microvascular endothelial cell growth medium-2, widely used in EPC culture. Systematic delivery of the growth factors is difficult with significant potential adverse effects such as hypotension, glomerular hypertrophy, and proteinuria. In comparison, sustained local delivery of these factors could represent a safer and more effective method of enhancing endothelial restoration.

PLGA particles and scaffolds have been widely used for controlled delivery of biomolecules including chemicals, proteins, and genes [35–37]. In contrast to other materials, such as hydrogels, which release hydrophilic proteins by rapid diffusion in a short-lasting burst, PLGA microparticles provide sustained drug delivery [38]. It is important to indicate that given degradation of these polymeric microparticles over time, their release efficiency may be between

20 and 40 % of the loaded biomolecules, a finding that is confirmed by our study [39].

In the present study, we used POC, which is a new stent coating polymer. An important feature of POC is that it possesses sufficient carboxyl groups which are essential for the formation of covalent coupling between POC, fibronectin, and anti-CD34 Ab [40]. PLLA, which is commonly used in stent constructs, on the contrary does not possess enough active carboxyl groups on its surface [9]. This limits conjugation of anti-CD34 Ab on its surface. This may explain our observation that conjugation of anti-CD34 Ab to PLLA did not appreciably improve EPC capture. Moreover, PLGA microparticle incorporation is also not feasible in PLLA as the PLGA microparticles dissolve in the solvent required for the preparation of PLLA coatings [41]. Our data show that the inability to incorporate growth factor containing microparticles into PLLA is an important limitation as the greatest increment in EPC proliferation on POC is observed with the inclusion of these microparticles within surface-enhanced POC. We also plan to further evaluate why untreated POC seem to have greater total cell DNA than untreated (or treated) PLLA.

In addition to POC, other biocompatible polymers such as PVDF-HFP fluorinated copolymer [42], phosphorylcholine polymer [42], polyurethane [43], and polycaprolactone [44] have been used for vascular stents or graft coating, with the objective of improved endothelialization. Others have combined sirolimus-delivering polymer abluminally along cell-capturing anti-CD34 Ab attached luminally on the stent struts [32]. Polymer-free stents with nanoporous surfaces have also been used in recent studies and have showed favorable endothelial recovery [45]. These studies indicate the immense interest in this area and different strategies that are currently being employed to improve endothelialization of implanted bioprosthetic devices.

Limitations

Although the modified POC coating shows good hemocompatibility, improved efficacy in capturing EPC under flow condition, as well as supports rapid EPC proliferation, it has important limitations. PLGA as a growth factor delivery vehicle can produce lactic acid and glycolic acid during its degradation [46]. Although they are considered to be natural metabolites of the human body, local accumulation of these acidic residues could be detrimental to the growth factors delivered by these particles and to neighboring tissues. Thus, additional studies need to be performed prior to the adoption of surface-enhanced POC polymer with PLGA microparticles for coating vascular prostheses to promote rapid healing. Other potential limitations of this study include a relatively small sample size, unblinded analysis of data, and use of EPC isolated from healthy donors. Despite these limitations, our results suggest a potentially new strategy for enhancing endothelialization of polymeric coatings of vascular prostheses.

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