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Regulation of human endothelial progenitor cell maturation by polyurethane nanocomposites

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ABSTRACT

The mobilization and homing of endothelial progenitor cells (EPCs) are critical to the development of an antithrombotic cardiovascular prosthesis. Polyurethane (PU) with superior elasticity may provide a mechanical environment resembling that of the natural vascular tissues. The topographical cues of PU were maximized by making nanocomposites with a small amount of gold nanoparticles (AuNPs). The nanocomposites of PU-AuNPs ("PU-Au") with a favorable response of endothelial cells were previously established. In the current study, the effect of PU and PU-Au nanocomposites on the behavior of human peripheral blood EPCs was investigated in vitro and in vivo. It was found that PU-Au promoted EPCs to become differentiated endothelial cells in vitro, confirmed by the increased expressions of CD31 and VEGF-R2 surface markers. The increased maturation of EPCs was significantly more remarkable on PU-Au, probably through the stromal derived factor 1α (SDF-1α)/CXCR4 signaling pathway. In vivo experiments showed that EPCs seeded on PU-Au coated catheters effectively reduced thrombosis by differentiation into endothelial cells. Surface endothelialization with CD31 and CD34 expression as well as intimal formation with α-SMA expression was significantly accelerated in the group receiving EPC-seeded PU-Au catheters. Moreover, the analysis of collagen deposition revealed a reduction of fibrosis in the group receiving EPC-seeded PU-Au catheters as compared to the other groups. These results suggest that EPCs engineered with a proper elastic substrate may provide unique endothelialization and antithrombogenic properties that benefit vascular tissue regeneration.

1. Introduction

Endothelialization is a protective mechanism with important functions, including antithrombosis, anti-inflammation, and inhibition of intimal hyperplasia [1]. Endothelial progenitor cells (EPCs) are considered as a promising alternative to primary endothelial cells for vascular tissue engineering [2]. These cells can be easily obtained from the peripheral or cord blood and have therapeutic potentials because of their proliferative and antithrombogenic properties. Asahara et al. characterized EPCs in human peripheral blood using magnetic bead selection in 1997 [3]. Mobilization of EPCs was observed in human patients with stroke [4] or myocardial infarction [5] to improve the function of ischemic tissues. An intramyocardial or intravenous injection of autologous EPCs increased vasculogenesis and improved cardiac function after myocardial infarction in animal experiments [6] and clinical trials [7]. EPCs combined with hydrogels have been implanted into various ischemic tissue animal models, for example, ischemic

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hindlimbs in mice [8] and areas of myocardial infarction in rats [9]. Abundant evidences suggest that EPCs contribute to vascular repair and remodeling.

Cells can respond to the substrate stiffness. Vascular grafts with low compliance can lead to smooth muscle cell overgrowth [10]. EPCs grown in an elastic scaffold encouraged the regeneration of vascular tissue [11]. Polyurethane (PU) is a popular biomedical elastomer and the rubber-like nature of PU may be matched to the microenvironment of vascular tissue [12]. The PU surface can possess favorable nanosized features by adding 5-nm gold nanoparticles (AuNPs) to make nanocomposites. Specially, PU-AuNPs nanocomposites ("PU-Au") with a relatively low concentration (~43.5 ppm) of AuNPs demonstrated superior in vitro and in vivo biocompatibility, which was believed due to surface alteration [13–18]. PU-Au coated commercial catheters significantly reduced thrombus formation in rat veins compared to PU coated or blank catheters [19]. Moreover, PU-Au can provide extracellular mechanical/topographical cues for cell migration. The migration of endothelial cells or mesenchymal stem cells is enhanced by up-regulation of the endothelial nitric oxide synthase (eNOS) expression via phosphoinositide 3 kinase (PI3K)/Akt signaling pathway [15,17] and Rho-GTPase expression via integrins/focal adhesion kinase (FAK) activation [18,20]. The above molecular signals are trigged by the cell–material interaction. Since PU and PU-Au are highly compliant materials, we hypothesize that they may provide a favorable microenvironment for the culture of EPCs. In particular, PU-Au with its unique surface features may further promote the migration and differentiation of EPCs.

Stem cell mobilization plays a critical role in cardiovascular regeneration [21]. The migration of EPCs and homing to ischemic tissues are mediated by cell adhesion molecules and chemokines [22–24]. Stromal-derived factor-1α (SDF-1α), which is constantly secreted by bone marrow stromal cells, immature osteoblasts, and endothelial cells [25], and its receptor CXCR4 are essential molecules that direct cell migration. The mobilization and angiogenic effects of EPCs are regulated by elevation of SDF-1α, which stimulates CXCR4 expression [26] for vascularization [27–29]. The role of SDF-1α/CXCR4 in modulating the response of EPCs seeded on a vascular implant, however, has not been reported so far. In this study, we investigate the interaction between EPCs and PU-Au nanocomposites, especially the activation of SDF-1α/CXCR4 by the materials. We hypothesize that the in vivo vascular repairing ability of EPCs upon the implanted materials may largely depend on the effect of the materials on the homing and differentiation behavior of EPCs.

2. Materials and methods

2.1. Preparation of polyurethane-gold nanocomposite films

The preparation of PU and the PU-Au nanocomposite followed that described earlier [15,16]. Briefly, the dispersion (50% solid content in distilled water) was synthesized based on hexamethylene diisocyanate (HDI) and poly(butadiene adipate) at a molecular ratio 3:1, and further chain-extended by ethylene diamine sulfonate sodium salt and ethylene diamine. A mixture of isocyanurate trimer of hexamethylene diisocyanate (HDI trimer) and 6% Bayer hardener (made from HDI trimer and polyethylene glycol) was added to the dispersion before casting into PU films. The average diameter of the AuNPs was 5 nm, determined by the transmission electron microscopy [16]. The PU-Au suspension was prepared to contain a concentration of 43.5 ppm of AuNPs in the final nanocomposite. Thin films of PU and PU-Au nanocomposites (PU-Au) were cast on 15-mm (Superior, Germany) or 32-mm round coverslip glass (Assistant, Germany) by a spin coater (Synrex PM-490, Taiwan). They were dried at 60 °C for 48 h and further dried in a vacuum oven at 60 °C for 72 h to remove any residual solvent. Surface characterization of PU and PU-Au including the surface morphology, roughness, and size domain was performed by the atomic force microscopy (AFM) (D3100; Digital Instruments, Veeco). AFM images were gained in the tapping mode in air with a triangular cantilever (force constant of 20–100 N/m) supporting a pyramidal tip of Si_3N_4 (PPP-RT-NCHR-50, Nansensors, Switzerland) [16]. The root-mean-square (RMS) surface roughness and the characteristic lateral size were calculated based on image analysis by the Image J software.

2.2. Purification and selection of $CD34^+$ human endothelial progenitor cells (EPCs)

Human blood samples were obtained with approval from the Institutional Review Board (IRB, Taichung Veterans General Hospital; IRB-TCVGH approval number CE12164). To obtain peripheral blood mononuclear cells (PBMCs), fresh blood samples of 250 ml were taken from young healthy adults. The blood sample was collected from three independent donors per week until the end of experiments. PBMCs were prepared from fresh or cryopreserved whole adult human peripheral blood sample. The layer of mononuclear cells was collected using the Ficoll-Histopaque (Sigma, USA) centrifugation method, and washed twice with 1 mM EDTA in phosphate buffered saline (PBS). The CD34-positive cells (EPCs) were enriched sequentially through positive selection using MiniMACS separation systems (Miltenyi Biotec, Bergisch Gladbach, Germany). In this system, immunomagnetic positive selection of EPCs was performed using paramagnetic microbeads each conjugated with anti-human CD34, followed by an anti-hapten antibody coupled with microbead, and were incubated with beads at ratios of 100 μl beads per 10^8 cells for 15 min at 4 °C. Flow cytometric analysis using anti-CD34 antibody (described in Sec 2.6).

2.3. Culture of adult EPCs and observation of morphology

EPCs were resuspended in 12 ml of RPMI medium supplemented with 1% (v/v) antibiotics (10000 U/ml penicillin-streptomycin), 2 mM glutamine, 10% FBS, and 50 ng/ml of vascular growth factor (VEGF) (Sigma, USA) (complete medium). Cells were seeded onto separate wells of a 6 well tissue culture plate precoated with fibronectin (0.05 mg/ml0) (Sigma, USA). After 24 h of culture in an incubator, non-adherent cells and debris were aspirated, and adherent cells were washed once with complete medium added. The medium was changed every 2–3 days for a total of 22 days. Cell morphology was monitored during this period by an inverted microscopy (Olympus, Lake Success, NY). EPCs appeared between 5 and 21 days of culture and were identified as well-circumscribed monolayers of cobblestone appearing cells. The endothelial cell phenotype at 21 days was examined by immunofluorescence staining (described in Sec. 2.5).

2.4. Proliferation assay of EPCs on different materials

PBMCs and EPCs were cultured in complete medium. PU and PU-Au on 15-mm coverslip glass were sterilized by 70% ethanol, rinsed and placed into the bottom of 24-well tissue culture plates (Corning, USA). One ml of cell suspension containing 2×10^4 cells was injected into each well of the culture plates. Cells cultured in a blank well (tissue culture polystyrene, TCPS) were used as control. For cell adhesion assay, cells cultured in a blank well (TCPS), as well as PU and PU-Au on 15-mm coverslip glass). After 24 h and 72 h of incubation, the adherent cells were trypinized and counted by using a hemocytometer combined with an inverted phase contrast microscope (Nikon TE-300, Japan). Besides, the adherent cells were harvested for MTT assay after 7, 14 and 21 days of incubation. Briefly, 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenyltetrazolium bromide (MTT) solution [(0.5 mg/ml, 1 × PBS] was added and incubated for 4 h at 37 °C. The supernatant was removed and the aliquot dimethyl sulfoxide (DMSO) (Tedia, USA) was added into each well to dissolve any resulting formazan crystals for 10 min. The absorbance was measured at 570 nm with a microreader (Molecular Devices, USA).

2.5. Immunofluorescence staining of endothelial cell phenotype on different materials

EPCs (2×10^4 cells per well) seeded in 24-well plates and incubated for 14 days were fixed with 4% paraformaldehyde and permeabilized with 0.5% (v/v) Triton X-100 in PBS for 10 min prior to staining. Following fixation and permeabilization, non-specific binding was blocked by adding 1% (w/v) bovine serum albumin (BSA) for 30 min. Cells were incubated in the primary anti-eNOS antibody solution (1:300 dilution, Santa Cruz, USA) and primary anti-vWF antibody solution (1:300 dilution, Santa Cruz, USA) for 60 min, washed extensively and then incubated with the appropriate secondary Cy5.5-conjugated immunoglobulin (red color fluorescence: anti-eNOS antibody) (1:300 dilution) or FITC-conjugated antibody (green color fluorescence: anti-vWF antibody) (1:300 dilution) for 60 min. Following further extensive washing, the nuclei were stained with 4′, 6′-diamidino-2-phenylindole (DAPI) (1:500 dilution) for 20 min. After further washes, coverslips were mounted on microscope slides with the storage solution (glycerol/PBS) and sealed with a synthetic mount gel. Images were gathered on a fluorescence microscope (Eclipse 80i, Nikon, Japan).

2.6. Flow cytometric analysis of surface markers for EPCs on different materials

EPCs (2×10^5 cells) were seeded into each well of the 6-well tissue culture plates containing 32-mm material-coated coverslips. Cells were labeled with fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies against CD34 (BD Pharmingen, USA), or phycoerythrin (PE) conjugated monoclonal antibodies against CD31 and VEGF-R2 (BD Pharmingen, USA). As negative controls, different FITC or PE conjugated isotype monoclonal antibodies (BD Pharmingen, USA) were used. Thereafter, cells were analyzed by a FACSCalibur flow cytometer (BD Biosciences, USA). Each assay included more than 10,000 events. Fluorescent cells were

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