Biomaterials 30 (2009) 3742–3748

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Enhanced dermal wound neovascularization by targeted delivery of endothelial progenitor cells using an RGD-*g*-PLLA scaffold

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ARTICLE INFO

Article history:
Received 19 January 2009
Accepted 21 March 2009
Available online 24 April 2009

Keywords:
Angiogenesis
Endothelial cells
Scaffold
Stem cells
Wound healing

ABSTRACT

Endothelial progenitor cells (EPCs), endothelial precursors that promote neovascularization in ischemic tissues, have shown the limited vascular regeneration efficacy due to their poor homing into injured sites and low survival, so that a variety of biosynthetic scaffolds have been employed as cell delivery vehicles to overcome the current cell transplantation methods. However, few paralleled studies that directly compare the efficacy of EPCs seeded within synthetic scaffolds to that of EPCs delivered by the conventional transplantation techniques used for EPC therapies have been performed. To address these issues, RGD-*g*-PLLA biosynthetic scaffold was developed for the targeted EPC delivery and was found to successfully support the *in vitro* growth and endothelial functions of EPCs. This scaffold also appeared to be good as *in vivo* targeted delivery carriers of EPCs as it promoted vascular regeneration in a murine dermal wound models. Furthermore, direct comparison with the intradermal EPC injection revealed that the targeted delivery of EPCs by using the RGD-*g*-PLLA scaffold was superior to their conventional local injection method in terms of the localization and survival/retention of the transplanted EPCs, and their vascular repairing potential. These results suggest that the development of an effective stem cell delivery system may help to maximize the tissue-repairing efficacy with a limited number of stem cells, thereby resolving the limited clinical success of current stem cell therapies that have utilized simple cell injections or infusions.

1. Introduction

Since endothelial progenitor cells (EPCs) were first identified as endothelial precursors that circulate in the blood, it has been shown by many studies that EPCs mobilized from bone marrow are recruited into injured tissues and contribute to neovessel formation by directly incorporating into vessel walls or by secreting a variety of angiogenic growth factors and subsequently stimulating angiogenesis [1]. Indeed, EPCs isolated from various sources including mobilized peripheral blood, bone marrow, cord blood, and adipose tissues, have been clinically proven to promote the postnatal neovascularization in diverse ischemic diseases [2,3].

However, several recent studies have shown that these therapeutic capabilities of EPCs and other stem/progenitor cells have not been fully utilized upon *in vivo* transplantation because of their poor bio-distribution and low cell survival. For example, it was shown that more than 90% of cells were entrapped at undesired organs including the liver, spleen, and kidney when they were intravenously injected [4]. Moreover, although some cells succeed in arriving at the ischemic tissues, a significant number of these cells undergo apoptosis before the injured tissues become ready for re-vascularization and relieved from ischemic shock [5]. To avoid these drawbacks of the systemic administration, cells have instead been injected directly at peri-infarct or infarct regions. Compared to systemic injection, this approach leads to significantly greater, but still marginal, number of injected cells that survive in the infarct

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doi:10.1016/j.biomaterials.2009.03.053

area and eventually contributed to the tissue regeneration [6,7]. Consistent with these reports, our previous study revealed that only a few cells out of intradermally injected EPCs participated in the dermal vascular regeneration at granulation tissues, whereas most of cells were retained at the site of injection in the skin [8].

To overcome these limitations of conventional transplantation methods, the present study aimed to develop a targeted EPC delivery system by using three-dimensional synthetic scaffolds that have a number of interconnective pores and large surface area for the cell in-growth. While a variety of synthetic polymers are available for use in biomedical applications, poly-L-lactic acid (PLLA) was chosen because of its biodegradable and relatively low cytotoxic properties and its surface was further modified to bear the Arg-Gly-Asp (RGD) peptide, a well-known synthetic ligand for integrin adhesion receptors, which promotes the cell adhesion, particularly by anchorage-dependent cells such as EPCs [9]. Since the RGD-grafted PLLA (RGD-*g*-PLLA) scaffold could provide a physical support of EPCs and also favorable environment for their cellular function, orthotopic transplantation of EPCs as being seeded within an RGD-*g*-PLLA scaffold was hypothesized to increase EPC localization and survival in hostile *in vivo* environments, and improve subsequent vascular regeneration. Here, we explored the potential of the RGD-*g*-PLLA polymeric scaffold serving as an EPC delivery carrier by examining the localization and retention of the implanted cells at injured tissues, and also evaluated its therapeutic superiority over the conventional local injection method in a setting of murine dermal wound healing model.

2. Materials and methods

2.1. Preparation of porous PLLA polymeric scaffold

Biodegradable porous PLLA ($M_w = 250{,}000$, Boehringer Ingelheim, Germany) scaffolds were prepared using gas foaming method as previously described [10]. The surface of the PLLA scaffolds was then modified through a graft-polymerization of hydrophilic monomer, acrylic acid (AA), using a radio frequency glow discharge device (Model PTS-003IDT, I. D. T. ENG., Inc., Seoul, Korea). The AA-grafted PLLA scaffold was immersed in the solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodimide (Sigma, St. Louis, MO, USA) for activation, and then in the 1 mM solution of Gly-Arg-Gly-Asp (Anygen, Seoul, Korea) for the peptide immobilization [9].

2.2. Seeding and in vitro culture of EPCs in RGD-g-PLLA polymeric scaffolds

EPCs were isolated by culturing CD34-positive cells from human umbilical cord blood in endothelial growth medium (EGM)-2MV Singlequot (Clonetics, Walkersville, MD, USA) for 2–3 weeks as previously reported [11]. The characteristics of these cells had been previously examined by our group, as shown by Supplementary Fig. 1 [cobblestone morphology; human CD31, KDR, and Tie2-positive expression; and tube formation on Matrigel (BD Bioscience, Bedford, MA, USA)-coated plate] [11]. The Institutional Review Board at Pochon CHA University approved all protocols, and informed consent was obtained from all donors. As shown in Fig. 1A, EPCs (2.5×10^5 in 20 μl of media) were loaded onto sterile disk-shaped RGD-*g*-PLLA scaffold (0.5 cm diameter, 1 mm thickness). After the complete adsorption of the EPC solution into the scaffolds, the scaffolds were cultured in EGM-2MV at 37 °C in a humidified incubator for 1 week.

2.3. Scanning electron microscope (SEM) analyses

EPC-seeded RGD-*g*-PLLA scaffolds were fixed in 2% glutaraldehyde and then in 1% osmium tetroxide (Sigma). After the serial dehydration in ethanol, samples were dried in a CO_2 critical point dryer and then were sputter-coated with gold for SEM analysis (SEM, JSM-5410LV, JEOL, Tokyo, Japan).

2.4. Proliferation assay and measurement of nitrite production

After seeding the same number of EPCs (2.5×10^5) into scaffolds or on tissue culture plate and culturing them in EGM2-MV, their proliferation was determined by using Cell-Counting Kit (CCK-8, Dojindo, Kumamoto, Japan) at the indicated time point (the first and seventh days after the seeding). Briefly, EPCs were incubated with 20 μl of the CCK-8 solution at 37 °C for 2 h, after which the absorbance at 450 nm was measured to estimate the cell numbers. The nitrite produced by EPCs was measured by using a commercially available Nitrite assay kit (R&D system, MN, USA) according to the manufacturer's instruction. Briefly, after cell culture medium was replaced with basal medium with or without the endothelial nitric oxide synthase (eNOS) inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME; Sigma), the EPCs were stimulated overnight with vascular endothelial growth factor (VEGF; 50 ng/ml). The supernatants were then analyzed to determine the nitrite level by employing a modified Griess reaction. Since NO mainly exists as nitrite or nitrate, the nitrate reductase enzyme was used to convert the nitrate into nitrite.

2.5. Transplantation and in vivo fluorescence imaging

6–8 Weeks old male athymic nude mice (Charles River Laboratories, Yokohama, Japan) were divided into group I (empty scaffold), group II (empty scaffold + intradermal injection of EPCs), or group III (EPC-seeded scaffold). The mice were then anesthetized with an intraperitoneal injection of ketamine–xylazine (79.5 mg/kg and 9.1 mg/kg, respectively), after which a full-thickness excisional wound (0.5 cm in diameter) was created on the dorso-lateral area by using a standard skin biopsy punch (Acuderm Inc., Fort Lauderdale, FL, USA). Immediately after surgery, an EPC-seeded scaffold (for group III mice) or an empty scaffold (for groups I and II mice) was implanted inside the wound. In addition, the group II mice were injected with EPCs (5×10^5 cells in 50 μl of PBS/wound) at three different intact dermis sites nearby the empty scaffold-implanted wound. The implanted scaffold was then covered with Tegaderm (3M, St. Paul, MN, USA), and secured in place with Surginet (One industry, Bucheon, Korea). To facilitate the *in vivo* optical imaging of the transplanted EPCs, the EPCs were labeled with fluorescent DiI (Molecular probes, OR, USA) prior to scaffold seeding (group III) and intradermal injections (group II). Their *in vivo* localization was imaged using the Xenogen IVIS Imaging System (Xenogen Coporation, Alameda, CA, USA) on day 7 after the scaffold implantation. To quantify the amount of DiI fluorescence in the tissues, the image data captured under a 488 nm excitation light were collected and their photon flux intensity was calculated and expressed as photons/second/cm^2 by using the IVIS imaging system software. All procedures were performed with the approval of Pochon CHA University Institutional Animal Care and Use Committee (IACUC).

2.6. Tissue preparation

Four weeks after dermal implantation, the scaffolds along with a 1 cm margin of the normal skin surrounding them were excised and prepared for following immunohistochemical staining and reverse transcriptase polymerase chain reaction (RT-PCR) experiments, as follows.

2.7. Quantification of vascularized area and immunohistochemistry

After fixation in 4% paraformaldehyde (Sigma), the harvested tissue samples were embedded and were serially sectioned (5 μm thickness) perpendicular to the wound surface. To assess dermal vascularization, the sections containing the central region of the implanted scaffolds were stained with hematoxylin and eosin (H&E) and photographed. The vascularized area in H&E-stained digitalized images was analyzed with NIH Image J analyzer by counting the pixels of the erythrocyte-filled vascular area and expressing it as a percentage of the total tissue area on the image.

To detect the presence of transplanted EPCs in the mouse vasculature, the sections were immunohistochemically stained with human CD31-specific antibody (DAKO, Carpinteria, CA, USA) and was visualized by using an ABC-alkaline phosphatase kit (Elite kit, Vector Laboratories, Burlingame, CA, USA) and Vector Blue (Vector Laboratories). For the experiment performed with DiI-labeled EPCs, the frozen sections were stained with 4,6-diamidino-2-phenylindole and imaged with a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Every 20th sections (i.e., at 100 μm intervals) around the central wound were subjected to vascularized area quantification and immunohistochemistry. The number of sections examined for following experiments ranges from 8 to 12 per wound.

2.8. Real time RT-PCR

Total RNA (1 μg) extracted from the harvested tissue samples was processed for cDNA synthesis by using the Superscript first-strand synthesis system (Invitrogen). Real time PCR was performed with SYBR-Green PCR master mix (Qiagen, Valencia, CA, USA) using Bioneer Exicycler™ 96 (Bioneer corporation, Daejeon, Korea). The specific primer sequences were as follows: human GAPDH, forward 5′-aagggtcatcatctctgccc-3, reverse 5′-gtgatggcatggactgtggt-3′: mouse GAPDH, forward 5′-atgactccactcacggcaaa-3′, reverse 5′-atgatgacccttttggctcc-3′. The relative level of hGAPDH mRNA was calculated by normalization for mGAPDH control in the sample. All reactions were performed in triplicate.

2.9. Statistical analysis

All data are presented as means ± SEM. One-way analysis of variance followed by Bonferroni's *post hoc* multiple comparison test was used to determine differences between groups. A p value of less than 0.05 was considered statistically significant and the number of samples examined is indicated by n.