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The role of fucosylation in the promotion of endothelial progenitor cells in neovascularization and bone repair

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ABSTRACT

Bone marrow-derived endothelial progenitor cells (EPCs) are being tested as a therapy to treat a variety of ischemic diseases. Poor homing to targeted tissues is one of the major factors limiting the therapeutic efficacy of EPCs. Here, we show that human cord blood-derived EPCs expressed little sialyl Lewis X (sLe^x) antigen that is necessary for selectin-mediated cell–cell interactions. Expression of α1,3-fucosyltransferase VI (FucT VI) in the EPCs enhanced sLe^x synthesis, E- and P-selectin-binding, and EPC adhesion to tumor necrosis factor-α-stimulated human umbilical vein endothelial cells in culture. In a mouse model of hind limb ischemia, in which EPCs were injected intravenously, FucT VI expression increased EPC homing, neovascularization, and blood flow in ischemic muscles. In another mouse model of femoral fracture, FucT VI-expressing EPCs were more efficient than control EPCs in targeting to peri-fracture tissues to enhance angiogenesis, blood flow and bone repair. These results indicate that fucosylated EPCs may be used to as an improved cellular source to treat ischemic diseases.

1. Introduction

Endothelial progenitor cells (EPCs) are bone marrow-derived stem cells capable of differentiating into endothelial cells [1–3]. The cells were first identified in adult human peripheral blood [4]. To date, EPCs have been isolated from bone marrow, umbilical cord blood and tissues such as liver and small intestine [2,3,5]. In general, EPCs are defined by surface markers, including CD34, CD133 and vascular endothelial growth factor receptor-2 (VEGFR-2), and phenotypes in culture, such as cobblestone-like appearance, colony formation, acetylated LDL (AcLDL) uptake, and tube-like structure assembly [2,3].

EPCs are able to migrate to injured tissues to promote angiogenesis and vasculogenesis. Because of the angiogenic and vasculogenic potential, EPCs have been exploited as a cell therapy to treat ischemic cardiovascular disease [2,5–10]. To date, more than 200 clinical trials involving EPCs have been registered, initiated or conducted in patients with heart disease, peripheral artery disease, aortic aneurysm, hypertension, diabetes, liver disease and cancer (ClinicalTrials.org). In these trials, isolated EPCs often were given intravenously. As such, the therapeutic efficacy largely depends on EPC homing to intended tissues. In several studies, however, only ~1–5% of intravenously injected EPCs/stem cells were found in injured tissues and incorporated into the vasculature [11–13]. The inefficient EPC homing in targeted tissues probably is one of the major reasons for the limited success so far in EPC trials [5,9,10,14]. Thus, strategies to enhance EPC homing are needed to improve EPC-based therapies.

Selectins are adhesion molecules that mediate cell–cell interactions [15–17]. E- and P-selectins, for example, promote leukocytes and platelets binding to activated endothelial cells, which are critical in inflammation, hemostasis, and wound repair [18,19]. The binding of selectins is mediated by carbohydrate determinants

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on their ligands. One such determinant is sialyl Lewis X (sLe^x), an α2,3-sialylated and α1,3-fucosylated tetrasaccharide on core-2 *O*-glycan [20–22]. In mice, lack of genes encoding α1,3-fucosyltransferase (FucT) and core-2 β1,6-N-acetylglucosaminyltransferase that are required for sLe^x synthesis impaired selectin-binding [23,24].

In addition to in leukocytes and platelets, sLe^x determinant participates in selectin-binding in other cell types. Previously, low levels of FucT expression and poor selectin-binding were reported in human cord blood-derived $CD34^+$ hematopoietic stem/progenitor cells [25,26]. Treating these $CD34^+$ cells with an exogenous FucT enhanced selectin ligand expression and promoted the cell engraftment in bone marrows [25,26]. The results indicate that sLe^x-dependent selectin-binding may contribute to adhesion and/or migration in specific subsets of hematopoietic cells.

EPCs and $CD34^+$ hematopoietic stem cells are believed to originate from common mesodermal precursors, *i.e.* hemangioblasts [1,27]. Thus, we hypothesized that a similar phenotype of low sLe^x antigen expression and poor selectin-binding may exist in EPCs and that enhancing sLe^x expression in these cells may improve the efficacy of EPC-based therapies in regenerative medicine. In this study, we tested the hypothesis in cell-based experiments and mouse models of limb ischemia and bone fracture.

2. Materials and methods

2.1. Human umbilical cord blood-derived EPCs

This study was approved by the ethics committee of the Second Affiliated Hospital of Soochow University. Written informed consents were obtained from blood donors. Heparin-anticoagulated cord blood was collected from healthy full-term deliveries. Mononuclear cells were isolated by density gradient centrifugation using Histopaque-1077 solution (Sigma–Aldrich). $CD34^+$ cells were purified from the mononuclear cells using an EasySep kit (StemCell Technologies) and cultured in human fibronectin-coated plates with endothelial cell basal medium-2 (Lonza) supplemented with EGM-2 MV SingleQuots medium containing 10% fetal bovine serum (FBS), human vascular endothelial growth factors, human fibroblast growth factor-B, human epidermal growth factor, R3 insulin-like growth factor-1, ascorbic acid, hydrocortisone, and antibiotics gentamycin (30 μg/mL) and amphotericin (15 ng/mL). After 48 h at 37 °C, non-adherent cells were removed by washing with phosphate-buffered saline (PBS) and fresh medium was added and replaced every other day. At sub-confluency, the cells were detached with trypsin-EDTA and cultured in new plates at 1:4 or 5 dilutions. Cells within 5 passages were used.

2.2. Flow cytometry

Flow cytometry was performed to analyze cell surface markers. Cultured EPCs were detached with EDTA and incubated with phycoerythrin (PE)-, fluorescein isothiocyanate (FITC)- or PerCP-labeled antibodies against CD14, CD31, CD34, CD45, CD105, CD146, CD133, CD144 or VEGFR-2 (BD Biosciences), which recognize the extracellular region of the targeted proteins. The antibody-labeled cells were analyzed by FACSCalibur (BD Biosciences).

2.3. Immunostaining

EPCs grown on slides were fixed with 4% paraformaldehyde (PFA). Immunostaining procedures were done, as previously described [28]. Antibodies against human VWF (DAKO), CD31 (DAKO) or CD144 (BD Biosciences) were used as primary antibodies and an Alexa Fluor 488-labeled antibody (Invitrogen) was used as a secondary antibody. Cell nuclei were stained with 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI).

2.4. RT-PCR

Total RNAs were isolated from EPCs, HUVECs, NIH-3T3 cells and peri-fracture tissues with an RNAeasy kit (Qiagen) and used to make cDNA templates. Human CD31, CD34, CD144, VEGFR-2, VE-cadherin and VWF mRNA expression was analyzed by RT-PCR using the oligonucleotide primers listed in Table S1 in Supplementary data.

2.5. AcLDL uptake and capillary tube formation assays

Cultured EPCs were incubated with Dil-labeled AcLDL (10 μg/mL) (Molecular Probes) in complete EGM-2 medium at 37 °C for 4 h. After washing with PBS, cells were stained with DAPI and examined for Dil-AcLDL uptake under a fluorescent microscope [29]. To test capillary tube formation, Matrigels (BD Biosciences) were added into 24-well plates and incubated at 37 °C for 30 min. EPCs in 200 μL of culture medium were plated onto the Matrigels, as previously described [30,31]. After 3 h at 37 °C, capillary tube formation was observed under a light microscope.

2.6. FucT VI expression in EPCs

The full-length human FucT VI cDNA was cloned into the pcDNA3.1 vector (Invitrogen) to yield plasmid pcDNA/FucT-VI. Recombinant FucT VI protein encoded by the plasmid contained a C-terminal V5 tag to facilitate protein detection. The plasmid and a control vector were transfected into EPCs by FuGENE reagents (Roche Diagnostics). Conditioned medium was collected after 48 h. The cells were lysed in a buffer containing 50 mmol/L Tris–HCl, pH 8.0, 150 mmol/L NaCl, 1% (v/v) Triton X-100 and a protease inhibitor cocktail (1:100 dilution, Sigma) [32]. FucT VI protein was analyzed by SDS-PAGE and Western blotting using an anti-V5 antibody (Invitrogen), as described previously [32].

2.7. sLe^x expression and selectin-binding assays

To measure cell surface sLe^x antigen, EPCs were incubated with an anti-sLe^x antibody (BD Biosciences) or control mouse IgM. Antibody-binding was detected with an FITC-conjugated secondary antibody by flow cytometry. For E- and P-selectin-binding, EPCs were incubated with human E- or P-selectin/IgG chimera (BD Biosciences) at 37 °C for 1 h. Bound E- and P-selectins on EPCs were detected by flow cytometry with PE-labeled anti-CD62E and FITC-labeled anti-CD62P antibodies (BD Biosciences), respectively.

2.8. EPC Adhesion to TNF-α–stimulated HUVECs

Confluent HUVECs in 96-well plates were stimulated with TNF-α (1 ng/mL) for 12 h. FucT VI- or vector-treated EPCs (3×10^4 cells) were added on top of the HUVECs in each well. After 1 h at 37 °C, non-adherent cells were removed by PBS washing. The remaining cells were fixed with 1% glutaraldehyde and stained with crystal violet. Total cells were quantified by measuring the OD at 570 nm in a plate reader. In a separated assay, Dil-AcLDL was used to label FucT VI- or control vector-treated EPCs. The labeled EPCs (3×10^4 cells) were added to the TNF-α-activated HUVECs at 37 °C for 1 h. After removing non-adherent cells, adhered cells were stained with DAPI. Dil-AcLDL-labeled EPCs adhered to HUVECs were counted in 3 randomly selected fields under a microscope.

2.9. Mouse models

Immune-deficient nude male mice (6–8 w; 25–30 g) were used in this study, which was approved by the Institutional Animal Care and Use Committee of the University. In a hind limb ischemia model [33], the proximal end of the right femoral artery was ligated above the origin of the circumflexa femoris lateralis. The mice were randomized into three groups and received tail vein injection with saline, control EPCs or FucT VI-EPCs, respectively.

In a femoral fracture model, a 3-mm medial parapatellar incision was made in the left leg. The patella was dislocated to expose the femoral condyles. A hole was drilled into the intracondylar notch and an intramedullary pin was inserted into the femoral canal till the proximal end. The patella was repositioned and the incision was closed. A closed diaphyseal fracture was introduced in the femur by a 3-point bending method [34]. The mice were randomized into saline, sham-control EPC and FucT VI-EPC groups. Two hour after the bone fracture was introduced, EPCs (1×10^6 cells in 100 μL saline) or saline were injected via tail vein. At different time points, mice were anesthetized for X-ray radiography and blood flow tests, and then euthanized for micro-CT and histological analyses.

2.10. EPC homing assays

FucT VI- or control EPCs (1×10^6 cells in 100 μL saline) were labeled with Dil-AcLDL and injected via tail vein. In the hind limb ischemia model, 3 days after EPC injection, gastrocnemius muscles were fixed in 4% PFA. Sections were made and examined under a fluorescent microscope. In the femoral fracture model, peri-fracture tissues were isolated 1 w post-surgery, fixed in 4% PFA and dehydrated with 20% sucrose. Sections (10 μm) were made, stained with DAPI and examined under a confocal microscope (Olympus FV500). Dil-AcLDL-labeled EPCs in the fracture sites were counted in 5 randomly selected fields and quantified using Image Pro-Plus software (Media Cybernetics).

2.11. Immunostaining blood vessels

In the hind limb ischemia model, 2 w after EPC injection, gastrocnemius muscle sections (6 μm) were fixed in 100% acetone and immunostained with an anti-CD31 antibody (BD Biosciences) [35]. In the femoral fracture model, periosteal tissues around the fracture sites were fixed in 4% PFA and immunostained with an anti-PECAM-1 antibody (BD Biosciences). Blood vessels in the sections were examined under a fluorescent microscope.

2.12. Blood flow assessment

Laser Doppler perfusion imaging (PIM 3 system, Perimed) was used to examine blood flow in injured tissues. Anesthetized mice were placed on a 37 °C platform and blood flow in tissues was monitored, as previously described [36]. In the hind limb