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Therapeutic Angiogenesis Using Genetically Engineered Human Endothelial Cells

Seung-Woo Cho[a,*], **Fan Yang**[b,*], **Sun Mi Son**[c], **Hyun-Ji Park**[a], **Jordan J. Green**[c], **Said Bogatyrev**[c], **Ying Mei**[c], **Sohyun Park**[d], **Robert Langer**[c,e,f], and **Daniel G. Anderson**[c,e,f,#]

[a]Department of Biotechnology, Yonsei University, Seoul 120-749, Korea

[b]Departments of Orthopaedic Surgery and Bioengineering, Stanford University, 300 Pasteur Drive, Stanford, CA 94304, USA

[c]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

[d]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

[e]David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

[f]Division of Health Science and Technology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

Abstract

Cell therapy holds promise as a method for the treatment of ischemic disease. However, one significant challenge to the efficacy of cell therapy is poor cell survival *in vivo*. Here we describe a non-viral, gene therapy approach to improve the survival and engraftment of cells transplanted into ischemic tissue. We have developed biodegradable poly(β-amino esters) (PBAE) nanoparticles as vehicles to genetically modify human umbilical vein endothelial cells (HUVECs) with vascular endothelial growth factor (VEGF). VEGF transfection using these nanoparticles significantly enhanced VEGF expression in HUVECs, compared with a commercially-available transfection reagent. Transfection resulted in the upregulation of survival factors, and improved viability under simulated ischemic conditions. In a mouse model of hindlimb ischemia, VEGF nanoparticle transfection promoted engraftment of HUVECs into mouse vasculature as well as survival of transplanted HUVECs in ischemic tissues, leading to improved angiogenesis and ischemic limb salvage. This study demonstrates that biodegradable polymer nanoparticles may provide a safe and effective method for genetic engineering of endothelial cells to enhance therapeutic angiogenesis.

Keywords

Polymer nanoparticles; Genetic engineering; Endothelial cells; Angiogenesis; Ischemia

[#]Correspondence to Department of Chemical Engineering, David H. Koch Institute for Integrative Cancer Research, and Division of Health Science and Technology Massachusetts Institute of Technology Building 76 Room 653, 77 Massachusetts Avenue, Cambridge, MA 02139, USA Tel.: 617-258-6843; Fax: 617-258-8827; dgander@mit.edu.
[*]These authors contributed equally to this work.

1. Introduction

Angiogenic cell therapy has been used to facilitate angiogenesis for treatment of ischemic diseases such as peripheral arterial disease or myocardial infarction [1-4]. Transplantation of endothelial cells (ECs) or endothelial progenitor cells (EPCs) have been reported to induce collateral blood vessel development in ischemic limb or myocardium [1-4], leading to improved limb survival or left ventricular function [1-4]. One challenge to angiogenic cell therapy is that ischemic regions are often exposed to hypoxic and inflammatory environments immediately after transplantation [5]. This environment can stimulate apoptosis, thereby limiting the efficacy of transplanted cells [5]. Indeed, significant levels of cell death have been observed after cell transplantation into ischemic tissue [6,7]. Several clinical studies have also reported that bone marrow-derived cell therapy provides little benefit to ischemic myocardium, in part due to poor cell survival in ischemic tissue [8,9].

Gene delivery has the potential to enhance the angiogenic efficacy of cell therapy by modifying cells with angiogenic or anti-apoptotic factors. For example, genetic modification of ECs or mesenchymal stem cells (MSCs) with the anti-apoptotic factor (Bcl-2) improved efficacy of cell therapy in a model of myocardial infarction or hindlimb ischemia [1,10]. Intramyocardial transplantation of MSCs transduced with adenovirus encoding vascular endothelial growth factor (VEGF) significantly enhanced angiogenesis and improved cardiac function of infarcted myocardium, compared with MSCs without VEGF modification [11]. However, most of studies have relied on viral vectors to deliver therapeutic genes to the cells, and thus raise the potential of safety concerns such as immunogenicity, mutagenesis, or toxicity [12]. Several studies have demonstrated the potential of non-viral vectors (polyethylenimine (PEI), FuGene, or Lipofectamine) for genetic modification [13-15], but the ability of those materials to transfect primary cells remains sub-optimal.

Poly(β-amino esters) (PBAE) are cationic polymers that hold promise as non-viral gene delivery for *in vitro* and *in vivo* application [16]. Conditions have been developed where PBAEs self-assemble with DNA to form stable nanoparticles that can effectively transfect a variety of cells, and biodegrade via hydrolytic cleavage of backbone ester groups [17]. High-throughput screening has identified lead PBAEs that are capable of transfecting several types of primary cells significantly higher than commercially available transfection reagents [18-21]. We reasoned that these biodegradable PBAEs could be used for *ex vivo* genetic engineering with angiogenic or anti-apoptotic factors to improve survival and engraftment of transplanted cells *in vivo* [22].

Here we develop human ECs for use in treating ischemic tissue in mice. Human umbilical vein endothelial cells (HUVECs) were transfected with VEGF using PBAEs, and then transplanted into ischemic limb tissue. We show that survival and engraftment of HUVECs in ischemic tissue is improved through polymer-mediated overexpression of angiogenic growth factors.

2. Materials and methods

2.1. Materials

Monomers were purchased from Sigma-Aldrich (St. Louis, MO), Scientific Polymer Products Inc (Ontario, NY), and Molecular Biosciences (Boulder, CO). Anhydrous dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich. Sodium acetate buffer solution (25 mM, pH 5.2) was prepared by diluting a 3 M stock solution (Sigma-Aldrich). $VEGF_{165}$ plasmid was kindly provided by Professor Johnny Huard at University of Pittsburgh and

amplified by Aldevron (Fargo, ND). The plasmid backbone is pBLAST49. pBLAST49-hVEGF$_{165}$ is a human VEGF expression vector containing VEGF165 sequence, blasticidin resistance gene, hybrid EF1α/HTLV promoter, and a multiple cloning site.

2.2. Poly(β-amino esters) (PBAE) synthesis

Acrylate-terminated C32 polymer (C32-Ac) was prepared by polymerizing butanediol diacrylate (C) with aminopentanol (32) using a 1.2:1.0 diacrylate to amine monomer molar ratio at 90°C for 24 hours (Fig. 1) [20]. Subsequently, amine-terminated C32 polymers were generated by reacting C32-Ac with diamine monomer (122) in DMSO (Fig. 1). End-capping reactions were performed overnight at room temperature using a 1.6-fold molar excess of amine over acrylate end groups.

2.3. Biophysical characterization

Particle size and zeta potential of the polyplexes were measured by using a ZetaPALS dynamic light scattering detector (Brookhaven Instruments Corp., Holtsville, NY) [18]. EGFP-N1 plasmid (Elim Biopharmaceuticals, Hayward, CA) was used for complexation. Samples were prepared for biophysical characterization in the same manner and at the same concentrations, as they were for transfection using 30:1 polymer/plasmid weight ratio. After a 10-min complexation, 200 μl of the polymer/DNA solution (n=3) was added to 1.4 ml of growth medium containing 12% serum to reach a final volume of 1.6 ml. Particle size and zeta potential was measured 1 h after complexation. Effective diameters were calculated from the autocorrelation function using the MAS option of the BIC particle-sizing software assuming a log normal distribution. The solution viscosity and refractive index were assumed equal to those of pure water at 25°C. Average electrophoretic mobilities were measured using BIC PALS zeta potential analysis software, and zeta potential was calculated using the Smoluchowsky model for aqueous suspensions.

2.4. Transfection

Human umbilical vein endothelial cells (HUVECs, Lonza, Walkersville, MD) were grown in Endothelial Growth Medium-2 (EGM-2) (Lonza) supplemented with the SingleQuot kit (Lonza) at 37°C and 5% CO$_2$. For viability test, HUVECs were seeded onto 96-well plates (15,000 cells per well) and transfected with VEGF plasmid (300 ng per well) in EGM-2 medium using optimized PBAE transfection conditions (1:30 (w/w) of plasmid to polymer ratio) [18]. The polyplexes were formulated by mixing PBAE with the plasmids in sodium acetate buffer and waiting 10 min for the complexes to form. The polyplexes were then added to HUVECs under EGM-2 medium with 12% fetal bovine serum (FBS). Following 2 hours of incubation, the medium with the polyplexes was replaced with fresh EGM-2 medium. Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) and polyethyleneimine (PEI), commercially available transfection reagents, were used for control transfection. Two days after transfection, cell viability (n=4) was measured using the CellTiter 96® Aqueous One Solution Cell Proliferation assay kit (Promega, Madison, WI). This kit employs a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity test. The tetrazolium compound contained in this kit is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by nicotinamide adenine dinucleotide phosphate or nicotinamide adenine dinucleotide produced by dehydrogenase enzymes in metabolically active cells. Cellular metabolic activity was determined by measuring optical absorbance at 490 nm using a Victor3 Multilabel plate counter (Perkin-Elmer Life Sciences, Boston, MA). For ELISA or real-time PCR analysis, HUVECs were seeded onto 6-well plates (3.0×10^5 cells per well) and transfected with VEGF plasmid (12 μg of plasmid per well, 1:30 (w/w) of plasmid to polymer ratio).

2.5. Enzyme-linked immunosorbent assay (ELISA)

Two days after transfection, the level of VEGF expression in HUVECs (n=3) was measured using an ELISA kit for human VEGF (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction. To obtain total proteins, cells were lysed in CelLytic™ MT (Sigma) containing a protease inhibitor cocktail (Sigma). After centrifugation of lysates, the supernatants were retrieved and used for protein samples. Total protein concentration in samples was determined by bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL). The VEGF expression was presented as the amount of VEGF per mg of total protein. The human VEGF expression was also examined in mouse ischemic limb muscle (n=3) retrieved at 2 days after HUVEC injection.

2.6. Quantitative real-time polymerase chain reaction (TaqMan method)

The mRNA expression of several molecules in HUVECs 2 days after transfection was examined using quantitative real-time polymerase chain reaction (PCR). Total RNA was isolated using the RNeasy Mini kit (Qiagen, Chatsworth, CA) from the cells of each group (n=4 per group). The RNA concentration was determined by measuring absorbance at 260 nm using a spectrophotometer. A reverse transcription reaction for cDNA synthesis was performed with 1 µg of pure total RNA using SuperScript™ III reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA). TaqMan® Fast Universal PCR Master Mix (Applied Biosystems) and synthesized cDNA were used for reaction. The expression of several molecules was quantitated using TaqMan® Gene Expression Assays (Applied Biosystems) for targets (PI3K: Hs00180679_m1, Akt-1: Hs00920503_m1, KDR/Flk-1: Hs00176676_m1, Tie-2: Hs00176096_m1, and VCAM-1: Hs01003372_m1) and endogenous reference (Glyceraldehydes 3-phosphate dehydrogenase (GAPDH): Hs02758991_g1). The expression level of target genes was determined by the comparative C_t method, whereby the target was normalized to the endogenous reference (GAPDH).

2.7. HUVEC culture under hypoxic and serum-deprived conditions

Two days after VEGF transfection, HUVECs were cultured under hypoxic and serum-deprived condition, a simulated ischemic condition [23]. EGM-2 was changed to Endothelial Basal Medium-2 (EBM-2) without FBS and growth factors, and then transfected HUVECs were further cultured for 2 days in a hypoxic incubator (MCO-18M, Sanyo, Japan) with air condition of 1% oxygen and 5% CO_2 at 37°C. Two days after culture, cell viability (n=4) was measured using the CellTiter 96® Aqueous One Solution assay kit. The viability of transfected HUVECs was converted to percent viability by comparison to that of HUVECs (without transfection) cultured under normal condition with normoxia (20% oxygen) and complete growth medium (EGM-2) for the comparable time period.

2.8. Hindlimb ischemia mouse model

All procedures for surgery were approved by "Committee on Animal Care" of Massachusetts Institute of Technology. Hindlimb ischemia was induced in a mouse model as previously described [24]. Female athymic mice at 6 weeks of age (18-20 g body weight, Taconic, Hudson, NY) were anesthetized with xylazine (10 mg/kg) and ketamine (100 mg/kg). The femoral artery and its branches were ligated through a skin incision with 5-0 silk suture (Ethicon, Somerville, NJ). The external iliac artery and all of the above arteries were then ligated. The femoral artery was excised from its proximal origin as a branch of the external iliac artery to the distal point where it bifurcates into the saphenous and popliteal arteries. All animals received humane care in compliance with "the Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH publication No. 85-23, revised 1996).

2.9. Cell transplantation

Immediately after arterial dissection, athymic mice were randomly divided into four experimental groups. The control group received an injection of only phosphate buffered saline (PBS, n=10). HUVECs (1.0×10^6 cells per mouse; no transfection, LIPO-VEGF, and C32-122-VEGF) were suspended with 100 µl of EGM-2 medium and injected intramuscularly into two sites of the gracilis muscle in the medial thigh using 29-gauge tuberculin syringes (n=10 per group). Physiological status of ischemic limbs was followed up to 4 weeks after injection treatments.

2.10. Triphenyltetrazolium chloride (TTC) staining

To identify the morphological differences among ischemic muscles in each group, the retrieved muscles (4 weeks after cell transplantation) were stained with TTC. The muscles were cut into 2-mm slices and then the slices were incubated for 30 min in 2% TTC (Sigma) solution and fixed for 30 min in 10% (v/v) buffered formaldehyde.

2.11. Histology

Ischemic and normal limb muscles were harvested at 4 weeks after cell transplantation. Specimens were fixed in 10% (v/v) buffered formaldehyde, dehydrated with a graded ethanol series, and embedded in paraffin. Specimens were sliced into 4-µm sections and the sections were stained with hematoxylin & eosin (H&E) to examine the muscle degeneration and tissue inflammation. Masson's trichrome collagen staining was performed to examine tissue fibrosis in the ischemic regions.

2.12. Immunohistochemistry

Tissue sections were prepared with ischemic limb muscles harvested 2 days and 4 weeks after cell transplantation. To detect transplanted human cells, the sections (from samples day 2) were immunofluorescently stained with anti-human nuclear antigen (HNA, Chemicon, Temecula, CA). Ten sections from two muscle samples of each mouse were randomly selected for the counts (3 mice per each group). For staining of capillaries in the ischemic regions, the sections (from samples week 4) were immunofluorescently stained with anti-von Willebrand Factor (vWF, Abcam, Cambridge, UK). The staining signals for HNA and vWF were visualized with rhodamine- and FITC-conjugated secondary antibodies (Invitrogen), respectively. The sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA) and examined using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). To quantify the number of HUVECs incorporated into vasculature, HNA- and vWF-stained cells were counted in ten sections from two muscle samples of each mouse (5 mice per each group). Microvessels for quantitative analysis were defined as mouse endothelial cell antigen (MECA)-positive microvessels. Tissue sections (from samples week 4) were immunohistochemically stained using antibodies against MECA (Chemicon). The staining signal was visualized with avidin-biotin complex immunoperoxidase (Vectastain® ABC kit, Vector Laboratories) and 3,3′-diaminobenzidine substrate solution kit (Vector Laboratories). Microvessels in ischemic areas were counted in ten sections from two muscle samples of each mouse (8 mice per each group) under a light microscope. Negative control staining was performed without primary antibody incubation.

2.13. Statistical analysis

Quantitative data are expressed as mean ± standard deviation. Statistical analysis was performed by the analysis of variance (ANOVA) using a Bonferroni test. A value of $p<0.05$ was considered statistically significant.

3. Results

3.1. Polymer nanoparticle synthesis

PBAEs were synthesized by the conjugate addition of amine monomers to diacrylates. Acrylate-terminated C32 (C32-Ac) was first synthesized by mixing butanediol diacrylate (C) with aminopentanol (32) in a 1.2:1.0 molar ratio (Fig. 1). C32-Ac was subsequently reacted with diamine monomers (122) to yield end-modified C32 polymers (C32-122) (Fig. 1). The nanoparticle complexation of polymer (C32-122) and plasmid DNA was confirmed by gel retardation assay (Fig. 2a). A transmission electron microscopic image showed the nanoparticle complexes of C32-122 and plasmid DNA with the size of around 200 nm (Fig. 2b). The particle size and zeta potential of PBAE nanoparticles were measured in 12% serum condition 1 hour after complexation with plasmid DNA. The particle size of C32 and C32-122 was 463 ± 35 nm and 219 ± 13 nm, respectively (Fig. 2c). Our previous study reported that the size of polymer-DNA nanoparticles increased over incubation time in 12% serum condition [18]. At 4 hours after complexation, the particle size of C32 and C32-122 increased to ~600 nm and ~400 nm, respectively [18]. The zeta potential of C32 and C32-122 was -1.91 ± 0.14 mV and -1.26 ± 0.19 mV, respectively (Fig. 2d).

3.2. Enhanced expression of VEGF and survival factors in vitro

Two days after VEGF transfection, VEGF expression was significantly enhanced (p<0.01) in HUVECs transfected with end-modified PBAE (C32-122), compared with no transfection, naked DNA transfection, or unmodified PBAE (C32) transfection (Fig. 3a). More importantly, C32-122 transfection (4.57 ± 0.37 ng/mg protein) significantly increased (p<0.01) VEGF expression, compared with commercially available reagents (PEI; 0.44 ± 0.03 ng/mg protein and Lipofectamine 2000; 0.71 ± 0.03 ng/mg protein) (Fig. 3a). Following VEGF transfection using C32-122, the expression of survival factors (anti-apoptotic factors, endothelial receptors, and adhesion molecules) was significantly enhanced in HUVECs (Figs. 3b-3f). Quantitative real-time polymerase chain reaction (PCR) analysis showed that the expression of anti-apoptotic factors (PI3K and Akt-1) was increased (p<0.01) in C32-122-transfected HUVECs, compared with no transfection or control transfection (C32 and LIPO) (Figs. 3b and 3c). C32-122-VEGF transfection also upregulated (p<0.01) the expression of endothelial receptors (KDR/Flk-1 and Tie-2) (Figs. 3d and 3e) and adhesion molecule (VCAM-1) (Fig. 3f). Expression of transgene (VEGF) in HUVECs was maintained up to 6 days after transfection (Fig. 3g). The duration of VEGF expression up to ~1 week could contribute to enhancing EC survival at early stage after transplantation into ischemic tissues.

3.3. Cell viability in vitro

VEGF transfection using PBAE (C32 and C32-122) did not cause significant cytotoxicity in HUVECs (Fig. 4a). However, similar conditions with 300 ngs of plasmid per 1.5×10^4 cells, Lipofectamine (1:1 ratio of plasmid to reagent) was more toxic to cells (p<0.01) than PBAE (1:30 ratio of plasmid to polymer) (Figs. 4a and 4b). When transfected HUVECs were cultured under a simulated ischemic condition with hypoxia (1% oxygen) and serum deprivation, the viability of C32-122-VEGF transfected HUVECs was much higher (p<0.01) than other HUVEC groups (Fig. 4c). The viability of HUVECs in C32-VEGF group is not significantly different (p>0.05) from that of HUVECs in no transfection group (Fig. 4c). The viability of LIPO-VEGF group is lower (p<0.05) than no transfection group, probably due to the cytotoxicity originated from Lipofectamine during transfection (Fig. 4c).

3.4. Enhancement of VEGF expression and cell survival in vivo

Genetically-engineered HUVECs were injected into mouse ischemic limb immediately after ischemia induction. The expression of human VEGF protein following HUVEC transplantation was enhanced by pre-transfection of C32-122-VEGF (Fig. 5a). Two days after cell injection, C32-122-VEGF group (620.8 ± 319.7 pg/mg protein) showed greater expression of human VEGF protein ($p<0.05$) in mouse ischemic muscle than no transfection group (82.0 ± 73.1 pg/mg protein), PEI-VEGF group (183.3 ± 116.2 pg/mg protein), or LIPO-VEGF group (204.6 ± 193.9 pg/mg protein) (Fig. 5a). Human nuclear antigen (HNA) staining of mouse ischemic muscle harvested at 2 days revealed that survival of transplanted HUVECs was improved by pre-transfection of C32-122-VEGF (Fig. 5d). The density of HNA-positive cells was higher ($p<0.01$) in C32-122 group than other control groups (Fig. 5b).

3.5. Angiogenesis promoted by genetically-engineered endothelial cells

Transplantation of HUVECs transfected with C32-122-VEGF significantly enhanced angiogenesis in mouse ischemic limb. Double immunofluorescent staining of HNA and mouse von Willebrand Factor (vWF) (Fig. 5e) showed that incorporation of transplanted HUVECs into host capillaries was more extensive ($p<0.05$) in C32-122-VEGF group than other groups (Figs. 5c and 5e). Immunohistochemical staining for mouse endothelial cell antigen (MECA, Fig. 6a) revealed that injection of HUVECs engineered with C32-122-VEGF promoted microvessel formation in ischemic tissue, compared with injection of phosphate buffered saline (PBS) or control HUVECs (no transfection, PEI-VEGF, and LIPO-VEGF). The density of MECA-positive microvessels in the ischemic region was significantly higher ($p<0.05$) in C32-122-VEGF group (120 ± 35/mm^2) than control groups (PBS; 40 ± 23/mm^2, no transfection; 50 ± 20/mm^2, PEI-VEGF; 60 ± 22/mm^2, and LIPO-VEGF; 78 ± 25/mm^2) (Fig. 6b).

3.6. Improvement of ischemic limb salvage by genetically-engineered endothelial cells

The therapeutic potential of genetically-engineered HUVECs was evaluated in terms of physiological status of ischemic limbs. The intramuscular transplantation of HUVECs engineered with C32-122-VEGF prevented necrosis of ischemic limbs. The injection of C32-122-VEGF-transfected HUVECs improved ischemic limb salvage (50%, 5 of 10), compared with either injection of PBS (10%, 1 of 10) or control HUVECs (no transfection; 20% (2 of 10), PEI-VEGF; 12.5% (1 of 8), and LIPO-VEGF; 30% (3 of 10), Fig. 7a). Ischemic muscle receiving HUVECs transfected with C32-122-VEGF has larger viable tissue area than other control groups, as confirmed by triphenyltetrazolium chloride (TTC) staining (Fig. 7a).

3.7. Reduced muscle degeneration following treatment with genetically-engineered endothelial cells

Histological examinations of the ischemic limbs retrieved at 4 weeks after transplantation confirmed that enhanced angiogenesis by transplantation of engineered HUVECs protected limb muscles against necrotic damage caused by ischemia. Hematoxylin and eosin (H&E) staining of the control group (PBS injection) showed muscle degeneration in the ischemic regions (Fig. 7b). In contrast, ischemic limb muscle injected with C32-122-VEGF-HUVECs was protected from necrosis (Fig. 7b). Masson's trichrome staining indicated that fibrosis was markedly attenuated after transplantation of C32-122-VEGF-HUVECs into ischemic region (Fig. 7b).

4. Discussion

Angiogenic cell therapy has therapeutic potential for a variety of diseases. Recently, it has been reported that cell therapy could be improved through genetic engineering of the cells to overexpress genes encoding for angiogenic growth factors or anti-apoptotic factors [1,10,11,13-15, 25]. Although this combined therapeutic strategy holds promise for the treatment of ischemic diseases, additional improvements to gene delivery are necessary to maximize the potential of this therapy. Viral vector-based delivery systems have demonstrated high efficiency of gene transfer, but they could raise serious safety risks of immunogenicity and genetic mutation which may hinder their clinical application [12]. Viral gene delivery is also plagued by production/manufacturing challenges and other limitations including nucleic acid cargo capacity [12]. Non-viral vectors may provide a safe approach to overcome current limitations of viral vectors. Numerous cationic polymers and lipids have been tested as vehicles for non-viral gene delivery including poly(L-lysine), PEI, DOPE, and others [26]. Those materials may confer several advantages including ease of preparation, purification and chemical modification as well as the stability. The transient nature of non-viral genetic modification may provide for additional safety advantages. While significant strides have been made in improving non-viral gene delivery, the efficacy is often sub-optimal, particularly for primary cells in the presence of serum [27].

We have developed end-modified PBAEs, easy-to-synthesize biodegradable polymers, that are able to deliver DNA to primary human cells [20]. Several end-modified PBAEs (C32-103, C32-117, C32-118, and C32-122) significantly enhance DNA delivery to various types of human stem cells (human MSCs, human embryonic stem cells, and human adipose-derived stem cells), showing a 2- to 5-fold higher transfection efficiency than commercially-available lipid reagent, Lipofectamine 2000 [18,19].

In the presence of 12% serum, end-modification of C32 polymer (C32-122) shows smaller particle size, more neutral zeta potentials, and improved stability [18]. VEGF transfection using end-modified PBAE (C32-122) significantly enhanced VEGF expression in HUVECs, compared with unmodified PBAE (C32), PEI, and Lipofectamine 2000 (Fig. 3a). In addition, C32-122 did not show significant cytotoxicity in gene transfer to HUVECs (Fig. 4a). Compared to Lipofectamine 2000, C32-122 was well tolerated in HUVECs for transfection with the DNA doses tested in our study.

Enhanced expression of endogenous VEGF might stimulate anti-apoptosis signal transduction in ECs. The expression of VEGF-dependent signaling molecules (PI3K and Akt-1) was enhanced by VEGF transfection with C32-122 (Figs. 3b and 3c). It is known that PI3K and Akt-1 signal transduction activated by VEGF could inhibit EC apoptosis [28,29]. VEGF is known to regulate the expression of endothelial receptors (KDR/Flk-1 for VEGF and Tie-2 for angiopoietin) and the signaling mediated by these receptors [30,31]. The expression of the endothelial receptors increased in HUVECs modified with C32-122-VEGF (Figs. 3d and 3e). The activation of those receptors may improve proliferation and angiogenic potential of ECs or EPCs [31,32]. These favorable changes in gene expression pattern led to improvement of the viability of HUVECs under a simulated ischemic condition with hypoxia (1% oxygen) and serum deprivation (Fig. 4c). Pre-transfection of C32-122-VEGF also promoted the survival of transplanted HUVECs in ischemic tissue at early stage of transplantation (Figs. 5b and 5d).

Genetic engineering using C32-122-VEGF facilitated engraftment of transplanted human ECs into mouse capillary network. It has been reported that enhanced expression of endogenous VEGF following transfection may increase expression of adhesion molecules (VCAM-1 and ICAM-1) [33]. Our study has shown that the expression of VCAM-1 was

upregulated in HUVECs following transfection with C32-122-VEGF (Fig. 3f). The enhanced expression of adhesion molecules in ECs may facilitate EC mobilization and incorporation into host vasculature [33,34]. Indeed, the incorporation of transplanted HUVECs into mouse vasculature was significantly enhanced by pre-transfection of C32-122-VEGF (Figs. 5c and 5e). Together with improved cell survival, efficient engraftment into host tissue greatly improved angiogenesis in ischemia regions (Figs. 6a and 6b). It has also been reported that overexpression of adhesion molecules (E-selectin, VCAM-1, or ICAM-1) in ECs can facilitate interaction between transplanted ECs and host EPCs, leading to enhanced neovascularization through recruitment of EPCs [35]. Enhanced blood vessel formation in ischemic tissue inhibited tissue necrosis and fibrosis caused by ischemic event (Fig. 7b) and ultimately improved ischemic limb salvage (Fig. 7a).

Combinations of molecules with different actions on angiogenesis may maximize the benefits of PBAE-engineered cell therapy. For examples, the combination of stromal-derived factor (SDF)-1α and VEGF significantly increased EPC-mediated angiogenesis in ischemic limb tissue [36]. Since SDF-1α is a chemokine for EPCs and VEGF increases the expression of SDF-1α receptors (e.g., CXCR4) on EPCs, this combination could enhance the efficacy of EPC therapy by promoting EPC recruitment to ischemic sites [36]. In other studies, bone marrow cell-based VEGF gene therapy combined with other angiogenic/myogenic factors (basic fibroblast growth factor or insulin-like growth factor-1) greatly enhanced angiogenesis and left ventricle function of ischemic myocardium [37,38]. PBAE-engineered cell therapy with multiple genes or combination with protein delivery could exhibit synergistic effects on therapeutic angiogenesis following cell transplantation.

Although this study has reported successful outcome of genetically engineered cell transplantation for therapeutic angiogenesis, the use of mature ECs needs be replaced with more potent cells such as EPCs. It is obvious that mature ECs (e.g., HUVECs used in this study) are good cell sources in experimental approach for demonstrating the effectiveness of VEGF transfection using PBAE nanoparticles. However, fully matured cells have some disadvantages for clinical application. Despite mature ECs such as HUVECs share some endothelial features with EPCs, they have a limited proliferative capacity, compared with EPCs. Thus, the use of mature ECs may lead to marginal therapeutic efficacy for treating ischemic diseases. In addition, ECs should be obtained from biopsy of autologous blood vessels, whereas EPCs can be easily isolated from autologous cell sources including bone marrow or peripheral blood, which are relatively more acceptable for patients with ischemia. This demonstrates the feasibility of EPCs for clinical setting. Indeed, recent clinical studies have focused on the application of CD34- or CD133-positive EPCs in ischemia treatments [39,40]. In this regard, genetic engineering using PBAE nanoparticles needs to be further evaluated for EPC transplantation for more clinically relevant therapeutic angiogenesis in future study.

This study suggests that genetically engineered ECs modified with non-viral DNA delivery systems may provide an effective system to promote therapeutic angiogenesis for the salvage of ischemic tissue. Genetic engineering of angiogenic cells could be applied for treatment of other types of ischemic diseases such as myocardial or cerebral ischemia. Since angiogenesis is an important process for most types of tissue regeneration, this technology may have utility in the repair of vascularized massive tissues including bone, skeletal muscle, or adipose tissue.

Acknowledgments

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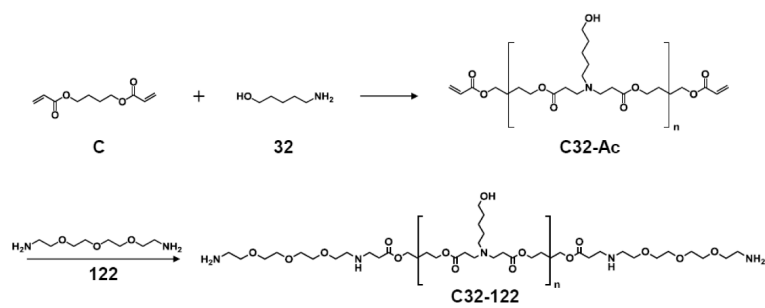
Fig. 1.
Synthesis of end-modified poly(β-amino esters). Acrylate-terminated C32 polymer (C32-Ac) was first synthesized and then end-modified with amine group (122).
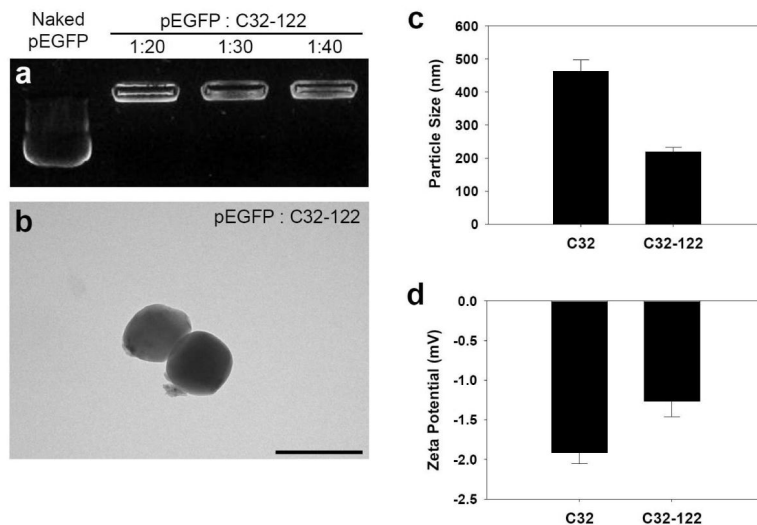
Fig. 2.
Characterization of the polyplexes of poly(β-amino esters) (C32 or C32-122) and DNA (EGFP plasmid). (a) Gel retardation analysis at 30 min after complexation. (b) Transmission electron microscopic image of the polyplexes. The scale bar indicates 200 nm. (c) Particle size and (d) zeta potential of the polyplexes measured by dynamic light scattering method at 1 hour after complexation.
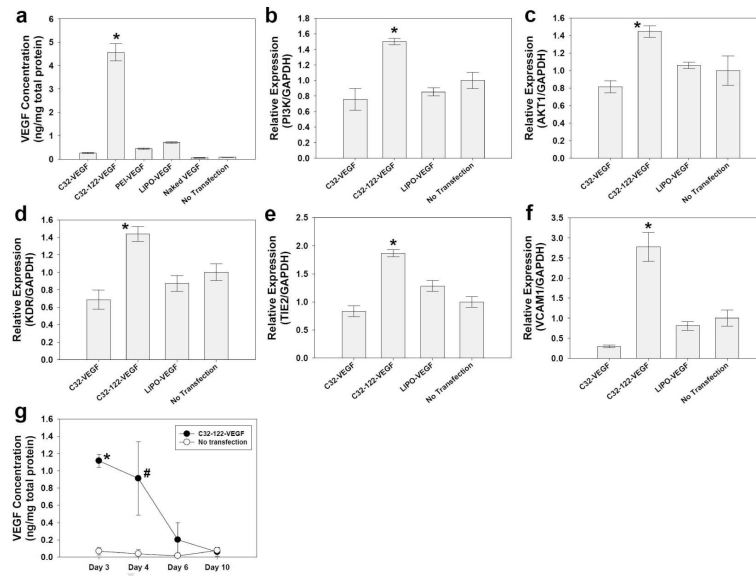
Fig. 3.
Enhanced expression of VEGF and survival factors in HUVECs 2 days after transfection. (a) ELISA for measurement of VEGF expression (n=3, *; p<0.01, compared to other groups). VEGF content was normalized to the amount of total protein. Real-time PCR for (b) PI3K, (c) Akt-1, (d) KDR/Flk-1, (e) Tie-2, and (f) VCAM-1 (n=4, *; p<0.01, compared to other groups). The relative expression of each molecule was normalized to no transfection group. (g) ELISA for examining the duration of VEGF expression in HUVECs at multiple time points (3, 4, 6, and 10 days) after transfection (n=3, *; p<0.01 and #; p<0.05, compared to no transfection group).
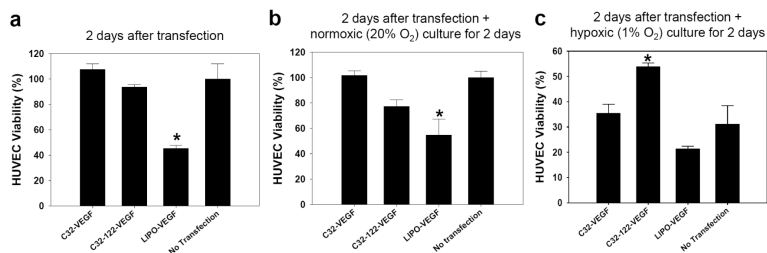
Fig. 4.
Viability of transfected HUVECs. (a) HUVEC viability 2 days after transfection (n=4, *; p<0.01, compared to other groups). The viability of transfected HUVECs was normalized to that of HUVECs without transfection. (b) Viability of transfected HUVECs 2 days after culture under normal culture condition (n=4, *; p<0.01, compared to other groups). Transfected HUVECs were recovered for 2 days after transfection and further cultured for additional 2 days in a normal condition with normoxia (20% oxygen) and complete growth medium (EGM-2). (c) Viability of transfected HUVECs 2 days after culture under hypoxia and serum deprivation (n=4, *; p<0.01, compared to other groups). Two days after transfection, HUVECs were transferred to hypoxic incubator (1% oxygen) with serum deprivation and further cultured for 2 days. The viability of hypoxic HUVECs was normalized to that of HUVECs (without transfection) cultured under normal condition with normoxia (20% oxygen) and complete growth medium (EGM-2) for the comparable time period.
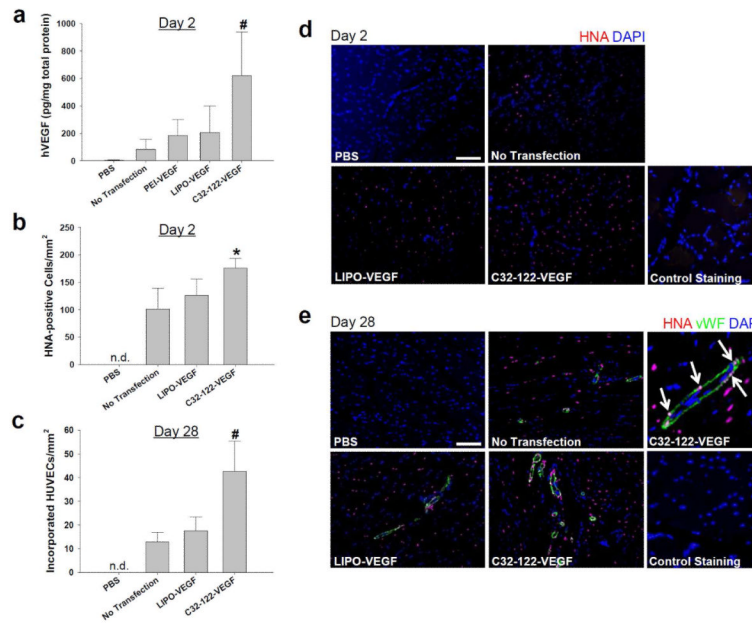
Fig. 5.
Enhancement of survival and engraftment of genetically-modified HUVECs in mouse ischemic limb muscles. (a) ELISA for human VEGF detection in mouse ischemic muscles (n=5) retrieved at 2 days after HUVEC injection (#; p<0.05, compared to other groups). Human VEGF content was normalized to the amount of total protein. (b) The density of HNA-positive cells (transplanted HUVECs) in mouse ischemic tissue 2 days after HUVEC injection (*; p<0.01, compared to other groups). (c) The density of HUVECs incorporated into mouse capillary in ischemic tissue 4 weeks after HUVEC injection (#; p<0.05, compared to other groups). (d) Immunofluorescent HNA staining of mouse ischemic muscle 2 days after injection. Scale bar indicates 100 μm. (e) Double immunofluorescent staining for HNA and vWF 4 weeks after injection. The colocalized signals of HNA and vWF (arrows) indicate transplanted HUVECs incorporated into mouse vasculature. Negative control staining was performed without primary antibody incubation. Scale bar indicates 100 μm.
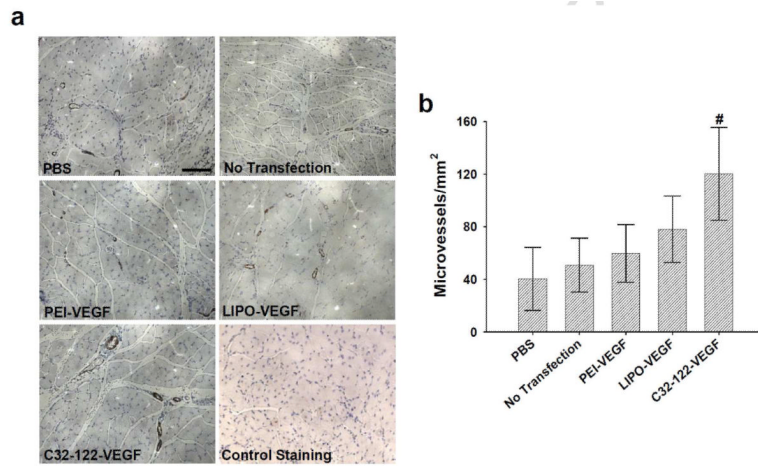
Fig. 6.
Enhanced angiogenesis by genetically-modified HUVECs in mouse ischemic limb muscle.
(a) Immunohistochemical MECA staining of mouse ischemic muscle 4 weeks after
injection. Scale bar indicates 100 µm. Negative control staining was performed without
primary antibody incubation. (b) Quantification of MECA-positive microvessel density in
ischemic regions. Microvessel formation was significantly enhanced by pre-transfection of
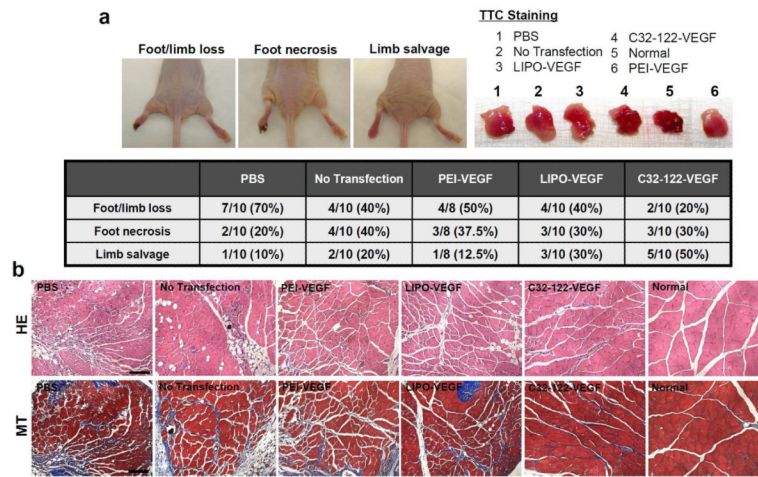C32-122-VEGF (#; p<0.05, compared with other groups).

Fig. 7.
Improvement of ischemic limb salvage by transplantation of genetically-modified HUVECs. (a) Physiological status of ischemic limbs 4 weeks after cell injection and TTC staining of ischemic muscles retrieved at 4 weeks after cell injection. (b) Histological analyses of normal limbs and ischemic limbs retrieved at 4 weeks after cell injection. H&E staining showed massive muscle degeneration in the ischemic region of control limb (PBS group). Transplantation of C32-122-VEGF-HUVECs prevented tissue necrosis in the ischemic region. Masson's trichrome staining indicated that fibrosis was markedly attenuated in the ischemic region by transplantation of C32-122-VEGF-HUVECs. Scale bars indicate 100 µm.