Transplantation of Nanoparticle Transfected Skeletal Myoblasts Overexpressing Vascular Endothelial Growth Factor-165 for Cardiac Repair

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Background—We investigated the feasibility and efficacy of polyethylenimine (PEI) based human vascular endothelial growth factor-165 (hVEGF165) gene transfer into human skeletal myoblasts (HSM) for cell based delivery to the infarcted myocardium.

Methods and Results—Based on optimized transfection procedure using enhanced green fluorescent protein (pEGFP), HSM were transfected with plasmid-hVEGF165 (phVEGF165) carried by PEI (PEI- phVEGF165) nanoparticles. The transfected HSM were characterized for transfection and expression of hVEGF165 in vitro and transplanted into rat heart model of acute myocardial infarction (AMI); group-1 = DMEM injection, group-2 = HSM transplantation, group-3 = PEI-phVEGF165-transfected HSM (PEI-phVEGF165 myoblast) transplantation. A total of 48 rats received cyclosporine injection from 3 days before and until 4 weeks after cell transplantation. Echocardiography was performed to assess the heart function. Animals were sacrificed for molecular and histological studies on the heart tissue at 4 weeks after treatment. Based on optimized transfection conditions, transfected HSM expressed hVEGF165 for 18 days with >90% cell viability in vitro. Apoptotic index was reduced in group-2 and group-3 as compared with group-1. Blood vessel density (×400) by immunostaining for PECAM-1 in group-3 was significantly higher (P<0.043 for both) as compared with group-1 and group-2. Improved ejection fraction was achieved in group-3 (58.44±4.92%) as compared with group-1 (P=0.004).

Conclusion—PEI nanoparticle mediated hVEGF165 gene transfer into HSM is feasible and safe. It may serve as a novel and efficient alternative for angiomyogenesis in cardiac repair. (Circulation. 2007;116[suppl I]:I-113–I-120.)

Key Words: nanoparticle ■ polyethylenimine ■ VEGF165 ■ skeletal myoblast ■ cardiac repair

Heart cell therapy using skeletal myoblast (SkM) transplantation limits postinfarction remodeling and improves global myocardial function.1-3 More recently, SkM transplantation is being combined with therapeutic angiogenesis by genetic modification of the donor cells to overexpress genes encoding for single or multiple growth factors.4,5 Though this combined therapeutic strategy holds great promise for the treatment of ischemic heart disease, the approach of transgene delivery into SkM needs to be optimized. Various viral vectors have demonstrated high transduction efficiency of therapeutic genes into SkM.4-7 Nevertheless, their use has demerits including immunogenicity and oncogenic potential which severely hinder their clinical application.8 Human clinical trials have shown that viral vector-based delivery of genes caused inflammatory reactions, formation of antiviral antibody, transient fever, and increase of liver transaminase.9-11 Nonviral vector gene delivery approach provides a safer alternative to overcome these untoward effects of viral vectors. Use of plasmid DNA either alone or complexed with cationic liposomes/polymers are being assessed with encouraging results.12,13 Similarly, the use of polymer based nanoparticles confers several advantages including ease of preparation, purification and chemical modification as well as their enormous stability.13,14 PEI has been widely used for nonviral transfection of cells.14 PEI is cationic in nature and with an intrinsic endosomolytic activity.14 All these properties of PEI contribute to its transfection efficacy.

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The present study has been carried out to design PEI (molecular weight 25 kDa; PEI-25) nanoparticles and optimization of conditions for transfection of hVEGF<sub>165</sub> gene into HSM with minimum cytotoxic effects. The genetically modified HSM were later used for heart cell therapy in an experimental rat heart model of AMI. We posit that the use of PEI nanoparticle for hVEGF<sub>165</sub> transfection of HSM is a safe and efficient approach for repair of the infarcted heart. We anticipate that nanoparticle gene transfection will envision a new approach for gene therapy in cardiovascular research.

**Methods**

**PEI-25 Complexation With Plasmid DNA**

Plasmids carrying enhanced green fluorescence protein (pEGFP) and phVEGF<sub>165</sub> were kindly provided by Dr Ratha Mahendran and A/Prof Ruowen Ge, National University of Singapore (NUS), respectively. Plasmid and PEI-25 solution (10 mmol/L) were diluted in 50 μL of 150 mmol/L NaCl separately. To determine optimum ratio between PEI and DNA, 15 to 24 equivalents of PEI nitrogen per DNA phosphate (N/P) were mixed using the following equation: N/P = (V × 10 mmol/L)/(Q DNA × 3); where V = volume (μL) of 10 mmol/L PEI and Q DNA = quantity of DNA (μg) used per 1×10<sup>6</sup> HSM. PEI-DNA complex was developed by mixing the respective saline solution containing DNA or PEI, and the mixture was vortexed gently followed by sedimentation for 10 minutes. PEI-DNA mixture was then used for transfection of HSM for 24 hours at 37°C.

**Loading Efficiency of PEI Nanoparticles**

The amount of encapsulated DNA in the PEI-25 nanoparticles was measured as the difference between the amount of plasmid DNA added to the nanoparticles and nonentrapped DNA. After complexation, the nanoparticle suspension was centrifuged for 15 minutes at 8×10<sup>4</sup> rpm and the supernatant was checked for the unbound DNA concentration with Nanodrop ND-1000 Spectrophotometer.

**Particle Size and Zeta Potential Analysis**

The size distribution and zeta potential of the PEI-DNA nanoparticle suspension were determined with a Zetasizer Nano ZS-machine (Malvern Instrument). The size distribution and zeta potential of the PEI-DNA nanoparticle suspension were determined with a Zetasizer Nano ZS-machine (Malvern Instrument). The size distribution and zeta potential of the PEI-DNA nanoparticle suspension were determined with a Zetasizer Nano ZS-machine (Malvern Instrument).

**Scanning Electron Microscopy**

Scanning electron microscopy was used to image the shape and size of the PEI-DNA nanoparticles. The buffer containing nanoparticles was spread on specimen stub (Agar Aid Scientific) and air-dried. The sample was coated with gold with a current at 15 mA for 30 seconds and then used for transfection of HSM for 24 hours at 37°C.

**Quantitative RT-PCR Analysis for hVEGF<sub>165</sub>**

Nontransfected, phVEGF<sub>165</sub>, and PEI-phVEGF<sub>165</sub> transfected HSM samples on 1, 4, 8, and 18 days after transfection were collected to quantify the hVEGF<sub>165</sub> gene expression. The following primers were used for hVEGF<sub>165</sub> expression (303bp): sense ATG AAC TTT CTG TCT TGG, anti-sense GTT GGA CTC CTC AGT GGG C; 18S was used as internal control and purchased from Ambion Inc (Catalog #1718). The isolation of total RNA and cDNA synthesis was carried out as described earlier. The QPCR thermal cycling program for 40 cycles was: 1 cycle of enzyme activation at 95°C for 15 minutes, denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 30 seconds.

**ELISA for hVEGF<sub>165</sub>**

Transfected HSM (2×10<sup>5</sup>) were grown on chamber slides for 24 hours and immunostained for hVEGF<sub>165</sub> expression as described earlier. immunoassays were used, HSM were transfected with PEI-pEGFP<sub>165</sub> using the optimized transfection conditions based on FACS results. The gene transfection and expression efficiencies were analyzed by immunostaining, RT-PCR, and enzyme-linked immunosorbent assay (ELISA).

**Immunostaining of HSM**

HSM transfected with PEI-phVEGF<sub>165</sub> were grown on chamber slides for 24 hours and immunostained for hVEGF<sub>165</sub> expression as described earlier. The immunostained cells were visualized using Olympus BX41 (Olympus) equipped for epifluorescence microscopy and images were recorded using a digital camera with MagnaFire 2.1 software.

**Cell Labeling**

One day before cell transplantation, HSM were labeled with 4, 6-diamidino-2-phenylindole (DAPI) (Sigma, USA) 12 hours at 37°C and 5% CO<sub>2</sub> incubator.

**Experimental Animal Model and Cell Transplantation**

All animals received human care in compliance with the Guide for the Care and Use of Laboratory Animals, NIH, USA and NUS. All animals were maintained by Animal Holding Unit of NUS. (Experimental AMI was induced in 48 young female Wistar rats (~200g, 10 weeks of age) by permanent ligation of left anterior coronary artery ligation. Ten minutes later, DMEM without HSM (group-1) or containing 1×10<sup>6</sup> nontransfected HSM (group-2) or PEI-phVEGF<sub>165</sub>transfected HSM (group-3) were intramyocardially injected into the infarct and perinfarct regions. All animals received cyclosporine (5 mg/kg/d) starting 3 days before and until 4 weeks after treatment. Sixteen animals were used for each group. Three animals per group were randomly sacrificed at 1 day and 1 week respectively. The remaining 10 animals in each group were sacrificed at 4 weeks after treatment.

Super-medium (Cell Transplants Singapore Pte Ltd). The purity and uniformity of HSM preparation was determined as described earlier.
Regional Blood Flow Studies
Rats harvested at 4 weeks (n=5 animal each group) after treatment received fluorescent microsphere injection to assess regional blood flow of left ventricle (LV). Under anesthesia, right or left femoral artery was isolated and prepared for blood sampling. One-milliliter solution containing 4×10^7 Fluospheres yellow-green polystyrene microspheres (Molecular Probes) was directly injected into LV while 4 mL blood sample was drawn. The microsphere from LV anterior wall and blood sample was recovered and regional blood flow was measured.4

In Situ Cell Death Assay
One day after cell transplantation, rat hearts (n=3 animal each group) were harvested to detect apoptotic cells in heart using In situ Cell death Detection Kit (Roche) as per supplier’s instruction. For apoptotic index, tissue sections were counter-stained with propidium iodide (PI) after TUNEL. Total number of cell nuclei and apoptotic nuclei were counted in four fields (×400) per slide, and the apoptotic index was calculated as the percentage of TUNEL+ apoptotic nuclei to total nuclei per field.

Immunohistological Studies
Tissue sections from hearts explanted at 1 week after cell transplantation were immunostained for hVEGF_{165} expression.4 The heart tissue harvested at 4 weeks after cell transplantation were immunostained for human skeletal myosin heavy chain (MY32, Sigma). Blood vessel density was measured at ×400 magnification in 8 microscopic fields/heart (n=5 animal each group) after double fluorescent immunostaining for PECAM-1 (Santa Cruz Biotechnology Inc) and smooth muscle actin (SMA; Sigma).

Heart Function Assessment
Echocardiography was performed by an investigator blinded to the therapeutic intervention on the animals using Aloka ultrasound machine (Aloka). From M-mode echocardiograms, measurements were obtained for LV-anterior wall thickness at end-diastole (LVAWTed), end-systole (LVAWTes), LV-internal diameters at end-diastole (LVIDed), and end-systole (LVIDes). LV-ejection fraction (LVEF) and fractional shortening (LVFS) were calculated as:

\[ \text{LVEF} = \frac{\text{LVIDes} - \text{LVIDed}}{\text{LVIDes}} \]

\[ \text{LVFS} = \frac{\text{LVIDes} - \text{LVIDed}}{\text{LVIDes}} \]

LV-anterior wall thickening percentage (LVAWTP) was calculated as:

\[ \frac{\text{LVAWTes} - \text{LVAWTed}}{\text{LVAWTed}} \]

Statistic Analysis
All statistical analyses were performed using SPSS (version 10.0). Apoptotic index, blood vessel density, and regional blood flow data were presented as median (25% quartile, 75% quatile) and analyzed.
by Friedman and Wilcoxon nonparametric methods to test any difference between the groups. The rest data were presented as mean ± SEM. Heart function between groups was analyzed by the method of analysis of variance (ANOVA) using Bonferroni test. All tests were performed with a significance level of 5%.

Statement of Responsibility
The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
Characterization of PEI-DNA Particles
The ultra structure of PEI-DNA complexes is shown in Figure 1A. The nanoparticle size and zeta potential showed concentration dependence (Figure 1B and 1C) and at higher N/P ratio, larger nanoparticle size and higher zeta potential were achieved. Loading efficiency of PEI-DNA nanoparticles was about 99% for all PEI-DNA ratios. PEI protected the encapsulated DNA from degradation for over 120 minutes as compared with the unprotected naked DNA which fully degraded after 30 minutes by DNase-I (Figure 1D).

Transfection of HSM With PEI-pEGFP
Maximum transfection of pEGFP (9.18 ± 2.37%) into HSM was achieved at 21:1 N/P ratio which was associated with low cell toxicity (cell viability = 92.48 ± 0.68%) (Figure 2A). Increase in DNA from 2 μg to 5 μg/1×10⁶ cells with N/P ratio at 21 also improved transfection efficiency (10.93 ± 3.1%) at 3 μg DNA (Figure 2B). The optimum transfection conditions using PEI-25 were N/P ratio of 21 using 3 μg DNA/1×10⁶ HSM and were used throughout the study (Figure 2C through 2E).

Characterization of PEI-phVEGF₁₆₅–Transfected HSM
Immunostaining revealed hVEGF₁₆₅ expressing HSMin vitro (Figure 3A and 3B). Transfection of HSM with phVEGF₁₆₅ resulted in poor transfection efficiency (1.32 ± 0.06 times as compared nontransfected HSM), whereas the gene expression of hVEGF₁₆₅ from PEI-hVEGF₁₆₅ transfected HSM increased 8.37 ± 0.21 times at day-2, 7.72 ± 0.27 times at day-4 and 2.84 ± 0.22 times at day-8, whereas it was 1.69 ± 0.04 times at day–18 (Figure 3C). ELISA showed that the transfected HSM secreted hVEGF₁₆₅ for 18 days of observation (4.47 ± 0.38 ng/mL) with peak level expression at day-2 after transfection (20.2 ± 1.51 ng/mL; Figure 3D).

Animal Studies
DAPI expressing HSM survived in the rat heart until 4 weeks after cell transplantation (Figure 4A and 4B).
HSM developed into multinucleated myotubes which stained positively for human skeletal myosin heavy chain (Figure 4C through 4E). The PEI-phVEGF165 myoblast also expressed hVEGF165 at 1 week after transplantation (Figure 4F through 4H).

Apoptosis
Apoptosis in infarcted area was reduced where HSM were transplanted (Figure 5C and 5D) as compared with DMEM injected group-1 (Figure 5A and 5B). This effect was further enhanced by hVEGF165 transfected HSM (Figure 5E and 5F). Though no significant difference ($P=0.109$) was achieved (because of the small animal number at 1 day after treatment), the percentage of TUNEL$^+$ cells in the scar zone was highest: 68.78% (62.16%, 77.32%) in group-1, whereas they were 39.75% (33.58%, 41.66%) in group-2 and 25.73% (24.41%, 28.33%) in group-3 (Figure 5G).

Evidence for Angiogenesis
Blood vessel density based on PECAM-1 immunostaining (at $400 \times$ magnification) was highest in group-3: 21.40 (18.69, 23.45; $P=0.043$) as compared with group-1: 9 (8.38, 10.75) and group-2 11.71 (10.47, 13.03) at 4 weeks after treatment (Figure 6A through 6I). The blood vessel density based on SMA immunostaining also was highest in group-3 15.3 (13.9, 18.13; $P=0.043$) as compared with group-1 and group-2. Dual immunostaining for PECAM-1 and SMA showed that percentage of the mature blood vessels in group-3 was 75.86% (72.26%, 77.29%) which was similar to those of group-1: 73.68% (62.5%, 77.5%; $P=0.5$) and group-2: 75.45% (69.32%, 78.28%; $P=0.893$; Figure 6L).

Regional Blood Flow
The LV-anterior wall blood flow (ml/min/g) was significantly reduced in group-1: 0.74 (0.5, 0.87) as compared with group-2 1.32 (1.13, 1.64; $P=0.043$) and group-3 2.3 (1.53, 2.84; $P=0.043$; Figure 7A).

Heart Function Studies
Echocardiography at 4 weeks showed that EF and FS in group-3 were 58.44$\pm$4.92% and 36.34$\pm$3.85%, respectively (Figure 7B and 7C). These were higher as compared with group-1 (41.67$\pm$4.77%, $P=0.004$ and 24.09$\pm$3.13%, $P=0.004$). Though EF (50.53$\pm$4.73%) and FS (30.2$\pm$3.26%) were improved in group-2, no significant difference was achieved as compared with group-1 ($P=0.432$ and $P=0.465$).

Though the LVIDed and LVIDes in all 3 animal groups increased, there was limited incremental tendency for
LVIDed and LVIDes in group-2 (6.63 ± 0.26 mm; P = 0.109; 4.64 ± 0.32 mm, P = 0.973) and group-3 (6.48 ± 0.23 mm, P = 0.109; 4.04 ± 0.26 mm, P = 0.043) as compared with group-1 (6.74 ± 0.41 mm; 5.18 ± 0.50 mm; Figure 7D and 7E). LVAWTed and LVAWTes were best maintained in group-3 (1.76 ± 0.27 mm; 2.72 ± 0.37 mm; Figure 7F and 7G), followed by group-2 (1.69 ± 0.44 mm and 2.41 ± 0.75 mm) and group-1 which showed thinning of LVAWTed (1.64 ± 0.33 mm) and LVAWTes (2.23 ± 0.53 mm). LV-anterior wall thickening percentage was highest in group-3 (56 ± 5.89%) followed by group-2 (43.06 ± 10.6%) and group-1 was the lowest (35.61 ± 6.49%) (Figure 7H).

Discussion

This study shows that transfection of HSM with PEI-phVEGF165 nanoparticles improves their reparability of the injured heart. High molecular weight PEI (800 kDa) has been used for gene transfer and forms compact and stable PEI-DNA complexes. However, this was associated with remarkably reduced cell viability. This effect can be moderated with low molecular mass PEI (5–48 kDa), whereas higher polymer concentration (increased N/P ratio) will be required to achieve comparable efficacy. N/P ratio and zeta potential dramatically influence the efficiency of PEI mediated gene delivery. Previous studies have shown that 5 to 6 NH2 nitrogen moieties of PEI are protonated at physiological pH and only these positively charged NH2 groups...
ionically interact with the negatively charged DNA. Our data are consistent with these observations.

Besides other factors, transfection efficiency is influenced by particle size which is mainly moderated by the molecular weight of PEI and N/P ratio. PEI-DNA complexes formed between 40 to 50 nm resulted in poorer transfection efficiency as compared with larger particle (200 to 300 nm). We observed that larger nanoparticle size gave higher transfection efficiency. However, for balance between cell viability and transfection efficiency, we opted for N/P at 21 as optimum transfection condition. We observed that incubation of HSM with PEI-DNA in the presence of low serum (2%) gave poor cell survival. Hence, we opted for Super-medium that contained 10% fetal bovine serum as gene transfection medium to promote cell viability during transfection reaction. Although 11% of HSM were transfected with PEI-hVEGF165, peak level expression (20.2 ng/mL) was observed on day-2 and a meager 4.47 ng/mL on day-18 after transfection. A comparison of the efficiency of PEI-25 with (n-[1-(2,3-Dioleoyloxy) propyl]-N,N,N-trimethylammonium methylsulfate) DOTAP for H9252-galactosidase gene delivery showed that when PEI-25 gave 48.3% A549 cells transfected and

Figure 7. A, Blood flow in group-3 showed significant improvement as compared with groups-1 and -2 at 4 weeks after cell transplantation (Error bar =25% to 75% quartiles). EF (B), FS (C), and LVIDes (D) in animal group-3 were significantly higher as compared with groups-1. Though LVIDed (E), LVAWTes (F), LVAWTed (G), and LVAWTP (H) were improved in animal group-3 and group-2, there was no significant difference was achieved between any groups. (B-H: error bar=SEM).

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expressed β-galactosidase protein (1.07±0.4 μg/μL). Contrary, DOTAP gave 49.3±2.7% A549 cells transected and expressed β-galactosidase only up to 0.47±0.3 μg/μL. A previous study has reported that >95% transected rat SkM expressed only 2.78±0.2 ng/mL hVEGF165 protein and was sufficient to initiate neovascularization. The initial hVEGF165 protein level in our study was 20.2 ng/mL from PEI-phVEGF165 transected HSM and 12.7 ng/mL during the first four days after transfection.

Our previous study using adenosival vector carrying hVEGF165 to transduce hSkM had resulted in more than 95% transduction efficiency. The peak level of VEGF protein (37±3 ng/mL) was reached at day-8 after transfection. Though the protein level in current study was lower than that, we believed that it was sufficient to initiate and maintain neovascularization. This was supported by the data of blood vessel density and LV regional wall blood flow in our study. Consistent with our previous studies, transplantation of PEI-phVEGF165 HSM had best improved LV systolic function and prevented LV remodeling. The surviving HSM not only expressed hVEGF165 to increase neovascularization, but also expressed skeletal myosin heavy chain in the rat heart. It was interesting that not only PEI-phVEGF myoblasts transplantation, but also skeletal myoblasts transplantation reduced apoptotic cells in cell transplanted area (Figure 5). This effect may be related with the paracrine factors released from HSM, including VEGF, hepatocyte growth factor, and platelet-derived growth factor. Moreover, overexpression of hVEGF165 from transected HSM was cytoprotective for cardiomyocytes in the ischemic myocardium in the acute stage, whereas it stimulated neovascularization and increased blood flow at later stage of infarction.

In summary, our study highlights the feasibility, safety, and efficacy of PEI-phVEGF165–transected HSM transplantation for cardiac repair. Reduced cardiomyocyte apoptosis, improved wall thickness, increased neovascularization, and regional blood flow of the infarcted myocardium together resulted in improved heart function. The PEI-25 based nanoparticle gene delivery approach may have clinical relevance and open a new concept for nonviral angiogenic gene delivery for the treatment of ischemic heart disease.

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Disclosures
None.

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