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Nanoparticle based delivery of hypoxia-regulated VEGF transgene system combined with myoblast engraftment for myocardial repair

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ABSTRACT

A regulated promoter system to control gene expression is desirable for safe and efficacious over-expression of therapeutic transgene. Combined with skeletal myoblast (SkMs), we report the efficacy of hypoxia-regulated VEGF gene delivery for myocardial repair during acute myocardial infarction (AMI). A hypoxia-regulated VEGF plasmid (pHRE-VEGF) was developed. After optimization, ~30% SkMs were transfected using polyethyleneimine (PEI) nanoparticles. The peak VEGF expression was higher in pHRE-VEGF transfected SkMs (^{VEGF}SkMs) under hypoxia (151.34 ± 8.59 ng/ml) than that with normoxia (16.92 ± 2.74 ng/ml). The efficacy of hypoxia-regulated gene expression system was assessed in a rabbit model of AMI. The animals were grouped to receive basal M199 without cells (group-1) or containing non-transfected SkMs (group-2) or ^{VEGF}SkMs (group-3). In group-4, ^{VEGF}SkMs were injected into normal heart to serve as normoxia control. Improved SkM survival was observed in group-3 and -4 ($p < 0.05$ vs group-2) at day-3 and 7 after transplantation. Blood vessel density was 20.1 ± 1.3 in group-3 which was significantly higher than any other groups ($p < 0.05$) at 2 weeks after treatment. Improved blood flow (ml/min/g) in the left ventricle (LV) anterior wall was observed in group-3 (1.28 ± 0.09, $p < 0.05$) as compared with group-1 (0.76 ± 0.05) and group-2 (0.96 ± 0.06), and similar to group-4 (1.26 ± 0.05). LV ejection fraction was best preserved in group-3 (58.4 ± 1.75%) which was insignificantly different from group-4 (61.1 ± 1.8%), and group-2 (52.8 ± 1.4%), but significantly improved compared with group-1 (44.7 ± 2.2%, $p < 0.05$). The study demonstrates that nanoparticle based delivery of hypoxia-regulated VEGF transgene combined with SkMs during AMI effectively preserves LV regional blood flow and contractile function of the heart.

1. Introduction

Vascular endothelial growth factor (VEGF) gene transfer to ischemic myocardium successfully initiates angiogenic response [1]. We have shown that transplantation of skeletal myoblasts (SkMs) transfected with polyethylenimine (PEI) nanoparticle carrying $VEGF_{165}$ into infarcted myocardium induced neovascularisation [2,3]. Given that myocardial ischemia is a recurring and often progressive disease and long-term uncontrolled over-expression of VEGF results in angioma formation [4], a more controlled and regulated expression of VEGF is expected to give better prognosis and abrogation of undesired effects. Therefore, the use of inducible and regulated gene expression systems is gaining popularity [5–8]. Some of the commonly used inducible systems include tetracycline operons, RU 486 (Mifepristone) and double oxygen–sensing vector system (using oxygen-dependent degradation domain).

Hypoxia inducible factor-1α (HIF-1α) is as a master regulator of multiple angiogenic growth factor expression in response to tissue hypoxia [9]. The expression of over 70 genes is regulated at the

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transcriptional level by HIF-1α, and the specific binding sites have been identified for many of these genes [9]. Hypoxia response element (HRE) has been reported in the 5′ or 3′ flanking regions of VEGF [10]. When HIF-1α binds to HRE under hypoxia, it up-regulates VEGF gene expression.

The combinatorial approach of SkM engraftment and angiogenic transgene delivery has been adopted to achieve synergy between angiogenesis and myogenesis for cardiac repair [3,11–13]. Preclinical studies have shown the effectiveness of SkM to form functional muscle fibers that improved contractile function and attenuated left ventricular remodelling [3,11–13]. Our studies on PEI nanoparticles for VEGF gene transfer into SkM demonstrated that PEI nanoparticles have high gene transfection and expression efficiency [2,3]. This non-viral angiomyogenic approach effectively induced neovascularisation and improved heart function in infarcted heart. It also overcomes the biosafety issues related to the use of viral vectors.

In the present study, we designed and manufactured one hypoxia-regulated VEGF plasmid (pHRE-VEGF) expression system, and investigated the efficacy of PEI nanoparticle mediated pHRE-VEGF gene transfer into rabbit SkM for repair of acute MI. We hypothesized that the use of PEI nanoparticle for pHRE-VEGF transfection of SkM is an efficient approach for improvement of donor cell survival during acute phase of cardiomyopathy, and increase angiogenesis and regional perfusion, and recover left ventricular function of the infarcted heart. We anticipated that this non-viral mediated pHRE-VEGF system would be a safe and efficient strategy for therapeutic angiogenic gene delivery for the treatment of ischemic heart disease.

2. Methods and materials

2.1. Preparation of pHRE-VEGF

Human $VEGF_{165}$ ($hVEGF_{165}$) was inserted into plasmid pGMT-easy (Promega, USA) and a fragment of 750 bp cDNA containing $hVEGF_{165}$ was cloned and inserted into pGL-3-Promoter Vector (Promega, USA) to replace luciferase gene. A 193 bp cDNA fragment containing 5x hypoxia response element (synthesized by Entelechon) was ligased into plasmid pGL-3 before SV40 promoter. Finally, a plasmid (~4.25 kb) containing $hVEGF_{165}$ (pHRE-$VEGF_{165}$) under the control of 5x HRE and SV40 promoter was derived.

2.2. PEI-22 nanoparticle complexation with pEGFP and pHRE-$VEGF_{165}$

PEI-22 was purchased from Euromedex (Souffelweyersheim, France), and filtered through a 0.22 μm filter. Plasmid and PEI-22 (5.47 mM) were diluted in 50 μl of 150 mM NaCl separately. To determine optimum ratio between PEI and DNA, 6 to 15 equivalents of PEI nitrogen per DNA phosphate were mixed according to the following equation: E = (V × 10 mM)/(QDNA X 3), where E = PEI nitrogen per DNA phosphate, V = volume (μl) of 5.47 mM PEI-22 and QDNA = quantity of DNA (μg) used per 1×10^5 SkMs [2,3].

The PEI-DNA complexes were developed by mixing the respective NaCl solutions containing DNA or PEI. After mixing, the mixture was vortexed for 30 s followed by sedation for 10 min. The loading efficiency of PEI nanoparticles was calculated and the physical shape was visualized by scanning electron microscopy while particle size and zeta potential were measured by a Zetasizer Nano ZS-machine (Malvern Instruments) [2,3,13]. PEI-DNA mixture was then used for transfection of SkMs for 24 h at 37 °C.

2.3. Isolation and culture of rabbit SkMs

Rabbit SkMs were isolated from the hind limb of male adult New Zealand White rabbits [14]. The site of skeletal muscle biopsy was stimulated with intramuscular injection of 0.5 mL of a mixture of ketamine (100 mg/mL) and xylazine (20 mg/mL). A muscle biopsy (1–2 g) was removed and processed under sterile conditions to isolate SkMs after 3 days. Rabbit SkMs were cultured in M199 medium supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, 100 μg/mL streptomycin, and 0.1 mg/mL L-glutamine. The cells were passaged every 2–3 days. Cells between passage 6–7 were used for gene transfection. The purity of isolated SkMs was determined by desmin expression.

2.4. Optimization of PEI-pEGFP transfection with rabbit SkMs

Trypsinized SkMs at a density of 1×10^5/well in 12-well plate were grown in 10% M199 and incubated with PEI-pEGFP complexes formed with equivalents (N/P) from 6 to 15. Transfection was continued for 24 h after which the transfection medium was replaced with fresh M199 medium. The transfected SkMs were characterized for gene transfection and expression efficiencies and viability by fluorescence activated cell sorting (FACS) in a Coulter flow cytometer (Epics Elite Esp, USA). SkMs with an adequate size and granularity were accounted for in the statistical analysis using non-transfected SkMs as a control to set baseline for auto-fluorescence limit [2,3,13]. Data were analyzed using WinMDI version 2.8 (Scripps Research Institute, USA) with gating at 1%.

2.5. SkMs transfection with PEI-pHRE-$VEGF_{165}$

SkMs were transfected with PEI-pHRE-$VEGF_{165}$ using the optimized transfection conditions based on FACS results. The gene transfection and expression efficiencies were analyzed by immunostaining, quantitative RT-PCR (QRT-PCR) and human VEGF Quantikine immunoassay kit (R & D Systems, Minneapolis, MN, USA) [3,13].

2.6. Cell labelling

One day before cell transplantation, SkMs were labelled with 4, 6-diamidino-2-phenylindole (DAPI) (Sigma, USA) that was diluted in 10% M199 for 12 h at 37 °C in humidified, 5% CO_2 incubator [3,13].

2.7. Acute myocardial infarction model and in vivo cell transplantation

All animals received human care and maintained by Animal Holding Unit of National University of Singapore (NUS). All animal procedures were conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and approved by the Institutional Animal Care and Use Committee (IACUC), NUS. All animals were monitored and maintained daily by staffs from Animal Holding Unit of National University of Singapore. Female rabbits (~2.5 kg) were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg), intubated and mechanically maintained by a small animal ventilator with a mixture oxygen (2 L/min) and isoflurane (2%). The heart was exposed aseptically through limited left sided thoracotomy, pericardium was removed. First branch of left circumflex coronary artery was permanently ligated using 4-0 prolene suture ($n = 19$/group). Ten minutes later, basal cell culture medium without cells (group-1) or containing 1×10^7 non-transfected SkMs (group-2) or ^{VEGF}SkMs (group-3) were intra-myocardially injected into the infarct area and peri-infarct area. To assess gene expression efficiency of this system under normal conditions in vivo, another 19 animals without MI received ^{VEGF}SkMs (group-4). The chest of the animal was closed and the animal was returned to cage and allowed to recover. All animals received cyclosporine treatment (5 mg/kg/day) from 3 days before and up to 2 weeks after surgery. Nine animals from each group were sacrificed on day-1, -3 and -7 after their respective treatment ($n = 3$ at each time point). The remaining 10 animals from each group were sacrificed at 2 weeks after surgery.

2.8. Blood flow study using fluorescent microspheres

Rabbits ($n = 5$ rabbits/group) at 2 weeks after treatment received fluorescent microsphere injection to assess regional blood flow as described [3,13].

2.9. Immunohistological studies

Immunohistological studies were performed on the heart tissue samples at 2 weeks ($n = 5$ rabbits/group) after cell transplantation for skeletal myosin fast heavy chain (A4335, Sigma USA) and myosin slow heavy chain expression (MAB1628, Chemicon USA) [3,13]. Blood vessel density was measured at 400 × magnification in 12 microscopic fields/heart ($n = 5$ rabbit/group) after double fluorescent immunostaining for PECAM-1 (CD31, Santa Cruz, USA) and smooth muscle actin (SMA) (Sigma, USA).

2.10. Quantification of male donor SkMs in rabbit heart

The survival rate of male rabbit SkMs in heart ($n = 3$ rabbits/group at each time point) at day-1, -3, -7 was determined by real-time PCR. The primers (for 237 bp fragment) for specific rabbit Y chromosome DNA are: sense: 5′-AATGTATGCCCTTAT GTTCG-3′, anti-sense: 5′-TTGCTGATGTCTGA GTTTCG-3′. Real-time PCR analysis was performed with the SYBR Green kit from Bio-Rad using DNA engine Opticon2 (Bio-Rad, USA) [2].

2.11. RT-PCR for pHRE-$hVEGF_{165}$ expression in vivo

The homogenized tissues were also used to isolate total RNA to determine the expression of pHRE-$hVEGF_{165}$ in heart at day-1, -3, -7. Total RNA was isolated using