Symposium: Laboratory Research

Skeletal myoblast based delivery of angiogenic growth factors: a comparison between angiopoietin-1 and VEGF gene delivery for therapeutic angiogenesis in the heart

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Objectives This study investigated the efficacy of human skeletal myoblasts (SkM) mediated either human vascular endothelial growth factor-165 (hVEGF165) or angiopoietin-1 (Ang-1) on vascular development and myocardial regional perfusion. Methods A porcine heart model of chronic infarction was created in 28 female swine by coronary artery ligation. The animals were randomized into: (1) group-1, DMEM injected (n=6), (2) group-2, Ad-null transduced SkM transplanted (n=6), (3) group-3, Ad-hVEGF165 transduced SkM transplanted (n=8), and (4) group-4, Ad-Ang-1 transduced SkM (n=8). Three weeks later, 5 ml DMEM containing 3×10⁶ SkM carrying exogenous genes were intramyocardially injected into 20 sites in left ventricle in groups-2, -3 and -4. Animals in group-1 were injected 5 ml DMEM without cells. Animals were kept on 5 mg/kg cyclosporine per day for 6 weeks. Regional blood flow was measured using fluorescent microspheres. The heart was explanted at 2, 6 and 12 weeks after transplantation for histological studies. Results Histological examination showed survival of lac-z expressing myoblasts in host tissue. Capillary density based on Von Willebrand factor-VIII (vWF-VIII) at low power field (>100) was 57.1±11.85 in group-3 at 6 weeks and declined to 32.1±5.21 at 12 weeks, while it was 39.9±10.26 at 6 weeks and increased to 45.1±6.54 at 12 weeks in group-4. The mature blood vessel index was highest in group-4 at 6 and 12 weeks after transplantation. The regional blood flow in the center and peri-infarct area was significantly increased in animals of groups-3 and -4. Conclusions SkM carrying either hVEGF165 or Ang-1 induced neovascularization with increased blood flow. Ang-1 overexpression resulted in mature and stable blood vessel formation and may be a more potent arteriogenic inducer for neovascularization. (J Geriatr Cardiol 2006;3:152-60.)

Key Words therapeutic angiogenesis; skeletal myoblasts; vascular endothelial growth factor-165; angiopoietin-1; myocardial infarction

Introduction

Extensive cell death and an associated myocardial dysfunction are the common features of chronic heart disease. Given the inadequate ability of the human heart to regenerate, a more recent approach to counter the remodeling process is to compensate for the loss of functioning cardiomyocyte number through stem cell transplantation with angiogenic potential. The novel approach of heart cell therapy is to repopulate the scar tissue with myogenic cells that may be functionally integrated into the host tissue. A wide variety of cell types, either genetically modulated or preprogrammed to adopt cardiac phenotype have been used for the treatment of damaged heart muscle. The ease of availability and that without ethical issues have greatly favored the use of SkM for cellular myocardial regeneration. SkM transplantation limits the expansion of infarcted left ventricle and reduces the remodeling progress to improve left ventricular contractile function.

One of the fascinating approaches in the field of cardiovascular gene therapy is to combine cell therapy with therapeutic angiogenesis. This approach will help to achieve neovascularization to enhance tissue perfusion in
the ischemic area along with neomyogenesis to strengthen the weakened heart muscle in the scar area. Vascular endothelial growth factor (VEGF), one of the most potent mitogens of endothelial cells, has been most widely studied for its angiogenic activity in experimental animal models. The advantage of VEGF therapy is improved blood vessel density, regional perfusion and induction of vascular protection. Despite the encouraging results from early phase I clinical trials of VEGF therapy, phase II controlled clinical trials demonstrated minimal clinical benefits. These results strongly implicate that VEGF alone may be insufficient to achieve clinically significant therapeutic angiogenesis for the treatment of ischemic heart. Hence a combination of growth factors and cells that participate at different stages of vascular development are needed to strengthen the therapeutic outcomes.

Angiopoietin-1 (Ang-1) has been assigned a pivotal role in angiogenic cascade. Despite Ang-1 having no mitogenic effect on endothelial cells, its disruption leads to distinct defects in vascular development. In vitro experiments showed that Ang-1 potently induced chemotactic response, network formation, sprouting, and survival in apoptosis without mitotic effect on endothelial cells. It is also an anti-permeable and anti-inflammatory agent in vitro and targets cell junctions by a decrease in basal permeability and inhibition of permeability responses to thrombin and VEGF. Compared with VEGF, Ang-1 is mainly involved in remodeling, maturation, and stabilization of blood vessels. Shyu et al. and Gurunluoglu et al. demonstrated that Ang-1 gene delivery promoted functionally neovascularization and perfusion. The increased perfusion was accompanied by the formation of more stable and mature vessels that were resistant to fluorescein isothiocyanate-conjugated albumin leakage. Thus, Ang-1 may be a better candidate for therapeutic angiogenesis. In current study, we investigated the efficacy of human SkM over expressing either human VEGF (hVEGF) or Ang-1 on vascular development and myocardial regional perfusion in a porcine heart model of myocardial infarction.

Methods

Culture of human SkM

The SkM were kindly provided by Cell Transplants Singapore Pte. Ltd. Singapore. The cells were cultured and propagated in 75-mm² tissue culture flasks and maintained with Supermedium (kindly provided by Cell Transplants Singapore Pte Ltd, Singapore) supplemented with 10% fetal calf serum (FBS) at 37°C in 5% CO₂ incubator until confluence. The purity and uniformity of human SkM preparation were assessed as described previously. The monocistronic adenoviral vector (Ad-vector) carrying hVEGF and Ang-1 were kindly provided by Dr Ruowen Ge, Department of Biological Sciences, National University of Singapore. The replication deficient Ad-hVEGF and Ad-Ang-1 were driven by immediate early human cytomegalovirus promoter. For propagation of the virus, HEK293 cells were cultured in DMEM supplemented with 10% FBS (10% DMEM). At 80-90% confluence, HEK293 cells were infected with virus and following full development of cytopathic effect, cells were harvested and lysed by 5 repeated freezing/thaw cycles. The cell lysate and the supernatant were purified using cesium chloride gradient ultra-centrifugation.

Transduction of human SkM with Ad-hVEGF or Ad-Ang-1

The cells were seeded at 1×10⁵ cells in 225-mm² tissue culture flasks and were exposed at a ratio of 1:1000 (cell: virus) for 8 hours as described. The viral infection medium was replaced with fresh growth medium after 24 hours. The transduction procedure was repeated three times to achieve optimum transduction efficiency.

In vitro characterization of the transgene expressing SkM

Fluorescent immunostaining The transduced SkM with Ad-hVEGF or Ad-Ang-1 or null adenoviral vector (Ad-null) were grown in glass chambers and fixed with -20°C cold methanol. The cells were incubated at 37°C with either mouse anti-VEGF or rabbit anti-Ang-1 primary antibodies (Chemicon Inc., USA). After washing, the cells were incubated with goat anti-mouse and goat anti-rabbit IgG conjugated with FITC or TRITC (Sigma, USA) respectively for 1 hour. The cells were visualized and counted in various microscopic fields for stained and unstained cells using a fluoresce microscopy (Olympus, Japan).

RT-PCR for hVEGF or Ang-1 gene expression For in vitro studies, SkM samples from non-transduced, Ad-null or Ad-hVEGF or Ad-Ang-1 transduced SkM (on days 1, 8, 18 and 30 after transduction) were collected. Total RNA was isolated using Total RNA Isolation Kit (QIAGEN, Germany) and RT-PCR was performed by using QIAGEN One-step RT-PCR Kit (QIAGEN). The primers for amplification were as follows: hVEGF (576bp) (forward) 5'-ATGAACTTTCTGCTG TCTTGGGTG 3' and (reverse) 5'-TCACGGCG TCGGCTGTCACA 3'; Ang-1 (378bp) (forward) 5'-CCGTGATAATTGCTGGGAAATGAG 3' and (reverse) 5'-GTAATGCTAC TTTATCCCATTCAG 3'; human GAPDH (305bp) (forward) 5'-AGCCACATCGCT CAGACACC-3' and (reverse) 5'-GTACTCGCCA CTATCCCCACATCAG 3'. For in vivo study, the primers of pig GAPDH (576bp) were (foward) 5'-TTCCAGGGCCACAGTCAA 3' and (reverse) 5'- GCAGGTCTAGTCCACAA 3', was used as house-keeping gene.

Animal studies

Animal groups and development of animal model Twenty-eight female swine, weighing 30±5 kg, were used in the preparation of the animal model and randomized into...
heart tissue samples were collected at 2, 6, and 12 weeks after transplantation. The animals were subjected to humane treatment. All procedures were carried out in accordance with the Institutional Protocols and Guidelines approved by the Animal Care and Use Committee of National University of Singapore and the Institutional Animal Care and Use Committee (IACUC), Singapore General Hospital, and assisted by a veterinary doctor. The animals were anesthetized and the heart was exposed by a limited left side thoracotomy. The pericardium was incised and a branch of the left circumflex coronary artery was ligated using 4-0 polypropylene suture. The successful occlusion of the blood vessel was confirmed by coronary angiography. The chest was closed and animal was maintained on cephalosporin for 4-5 days for prophylactic treatment of wound infection.

Transplantation of SKM and transient immunosuppression For post-transplant identification, SKM were transduced with retroviral vectors carrying Lac-z reporter gene with nuclear localization signal. Cell viability prior to transplantation was >99% as determined with 0.2% trypan blue. Three weeks after the establishment of animal model, the animal was prepared for a second left side thoracotomy as described earlier. Five milliliters of basal DMEM without SKM (group-1) or containing 3×10^6 SKM transduced with either Ad-null (group-2), or Ad-hVEGF_165_ (group-3), or Ad-Ang-1 (group-4) were intra-myocardially injected at 20 different sites in and around the infarct area. The animals received 5mg/kg/day cyclosporine from 5 days prior to until 6 weeks after cell transplantation.

Histological studies The animals were euthanized and heart was explanted. The cell injection site of the myocardium was sectioned into 5-mm thick cross-sectional pieces along the longitudinal axis. Thin cryosections of 6-8 μm thickness were cut and stained for Lac-z expression as described earlier. Lac-z positive tissue sections were immunostained using antibodies with various specificities including myosin heavy chain (slow and fast isoforms), connexin-43, smooth muscle actin, and von Willebrand factor-VIII (vWF-VIII).

PCR for human Y-chromosome in pig heart Porcine heart tissue samples were collected at 2, 6, and 12 weeks after SKM transplantation. DNA was isolated from the samples according to manufacturer’s instruction using DNeasy-Tissue Kit (QIAGEN, Germany). TaqPCR Master Mix Kit (QIAGEN, Germany) was used in PCR for human Y-chromosome. Primers for PCR amplification of human Y-chromosome were (forward) 5'CATGAAAGCGATTCCATCTGTTGGTC 3' and (reverse) 5'CTGGGGAAGCACAATGCAATTT 3'; primers for pig γ-actin were (forward) 5'GGAAGCTCTGCTATTGGAGT 3' and (reverse) 5'TGTCGTITTCTGAGAACAGG 3'.

Regional blood flow measurement Fluorescent microspheres (Molecular Probes, USA) with three different spectra were injected (1.4×10^4 microspheres/kg body weight), at 0, 6 and 12 weeks post SKM transplantation. Arterial reference blood samples were obtained from external femoral artery at a constant rate of 10 ml/min. Animals were euthanized at stipulated time intervals and tissue samples and reference blood samples were processed to extract the fluorescent dye. The fluorescence activity was measured using a Perkin Elmer LS-50B spectrophotometer. Regional myocardial blood flow (Q, ml/min/g) was calculated as Q = (fl/ftref)×R, where fl and ftref are fluorescence of the tissue sample and the reference blood sample respectively, and R is the withdrawal speed of arterial reference sample (ml/min).

Statistical analysis Statistical analysis was performed by SPSS 11.0. All data were presented as mean ± standard deviation (SD) and analyzed by analysis of variance (ANOVA). P<0.05 was considered statistically significant.

Results

Characterizations of cultured human SKM

The cultured SKM was more than 98% pure for desmin expression (Fig. 1). Immunohistochemical staining of Ad-hVEGF_165_ or Ad-Ang-1 transduced SKM revealed high transduction efficiency of the transgenes (Figs. 2a & 2b). Fluorescent immunostaining showed that more than 95% of SKM were transduced and expressed hVEGF_165_ or Ang-1. Ad-null transduced SKM were used as a negative control (Fig. 2c). RTPCR for hVEGF_165_ or Ang-1 expression revealed that the transduced SKM expressed hVEGF_165_ and Ang-1 transgenes for up to 30 days of observation in vitro (Fig. 3). The peak expression level was achieved at day 8 after multiple transductions.

Animal study

All animals survived the full length of experiment. There was no macro- or microscopic evidence of tumor development in any of the cell transplanted animal heart. Immunosuppression was successfully achieved using 5mg/kg cyclosporine per day starting 5 days prior to for up to 6 weeks after cell transplantation. The animals were killed at 2, 6 and 12 weeks after cell transplantation. The heart tissue was explanted,
Fig. 2. (a) Immunostaining of Ad-hVEGF<sub>165</sub> transduced SkM for hVEGF<sub>165</sub> expression (blue fluorescence= hVEGF<sub>165</sub> expression). (b) Immunostaining of Ad-Ang-1 transduced SkM for Ang-1 expression (red fluorescence= Ang-1 expression). (c) Ad-null transduced SkM as a negative control. Cells were counter-stained with DAPI to visualize nuclei (blue fluorescence= DAPI).

and the site of injection was processed for tissue RT-PCR, histochemical and immunohistochemical analysis.

Histochemical examination of pig heart tissue for Lac-z expression revealed extensive survival of SkM in pig heart after myoblast transplantation (Fig. 4a). Immunosuppression was discontinued at 6 weeks after SkM transplantation. The survival of the donor SkM was further confirmed by PCR for human Y-chromosome in the pig heart (Fig. 4b). RT-PCR of pig heart for hVEGF<sub>165</sub> or Ang-1 expression further demonstrated that transplanted SkM actively expressed hVEGF<sub>165</sub> or Ang-1 at the site of the cell graft (Figs. 5a & 5b). The transgene expression continued in vivo at least for 2 weeks and which was significantly reduced at 6 weeks.

Dual fluorescent immunostaining for vWF-VIII (for endothelial cells) and SMA (for smooth muscle cells) suggested that angiogenic factors, delivered by SkM, efficiently increased blood vessel density in the cell transplanted region at 6 and 12 weeks after cell transplantation (Figs. 6 & 7). Blood vessel density (number of blood vessels per microscopic field) based on vWF-VIII expression at low power (100×) was 16.18±2.56 at 6 weeks in group-1 which decreased to 13.44±2.82 at 12 weeks (Fig. 8a). For group-2, blood vessel density was 26.57±5.53 and 26.86±5.7 at 6 and 12 weeks, respectively. The highest blood vessel density was achieved by hVEGF<sub>165</sub>-myoblast transplantation at 6 weeks (57.13±11.85). However, it

Fig. 3. RT-PCR for (a) hVEGF<sub>165</sub> and (b) Ang-1 expression from Ad-VEGF<sub>165</sub> or Ad-Ang-1 transduced SkM
Lane 1= DNA ladder; Lanes 2 & 3= Non-transduced SkM; Lanes 4 & 5= Ad-null transduced SkM; Lane 6-13= Ad-VEGF<sub>165</sub> or Ad-Ang-1 transduced SkM at 1, 8, 18 and 30 days after transduction

Fig. 4. (a) Histochemical staining for Lac-z expression in pig heart showed the survival of SkM in pig heart up to 12 weeks after cell transplantation. (b) PCR for human Y-chromosome in pig heart tissue at 2, 6 and 12 weeks after myoblast transplantation using DMEM injected pig heart tissue as a negative control and pig γ-actin as house-keeping gene (c).

Lane 1= DNA marker; Lane 2= Human Y-chromosome from human male SkM as a positive control; Lanes 3, 6, 9, 12= Center of infarction; Lane 4, 7, 10, 13= Peri-infarction; Lane 5, 8, 11, 14= Remote away from infarction.
Fig. 6. Visualization of blood vessel in pig heart (magnification 100×) at 6 weeks after cell transplantation. Dual fluorescent immunostaining for vWF-VIII (red fluorescence= TRITC) and SMA (green fluorescence= FITC) was carried out to visualize blood vessels.
Fig. 7. Visualization of blood vessel in pig heart (magnification 100x) at 12 weeks after cell transplantation. Dual fluorescent immunostaining for vWF-VIII (red fluorescence=TRITC) and SMA (green fluorescence=FITC) was carried out to visualize blood vessels.
significantly declined to 32.1±5.21 (P=0.001) at 12 weeks. The progressive increase in blood vessel density was only seen in group-4 where it continuously increased from 39.9±10.26 at 6 weeks to 45.14±6.54 (P=0.365) at 12 weeks. For inter-group comparison, vWF-VIII immunostaining showed that the blood vessel density of group-3 and group-4 were significantly higher than those of groups-1 and -2 at 6 weeks (P<0.001) and 12 weeks (P<0.005).

The blood vessel density based on SMA immuno-staining demonstrated that mature blood vessel density in groups-1 and -2 remained almost unchanged at 6 weeks (7.88±1.46 and 20.14±4.45 respectively) and 12 weeks (6.87±1.81 and 19.29±7.59 respectively) (Fig. 8b). However, it increased in groups-3 and -4 from 20.25±3.81 and 34.73±8.34 at 6 weeks to 29±5.52 (P=0.02) and 41.36±5.72 (P=0.06) at 12 weeks respectively. The highest mature blood vessel density was achieved in group-4 at 6 and 12 weeks (Fig. 8b) and was significantly higher than any other group (P<0.001). Although mature blood vessel density of group-3 increased to 29±1.84 at 12 weeks and was significantly higher than that of groups-1 and -2 (P<0.01), it was significantly less than that of group-4.

The mature blood vessel index showed that group-4 had the highest index number and this progressively increased from (85.26±9.47)% at 6 weeks to (91.64±2.98)% (P=0.32) at 12 weeks. However, it remained unchanged at 6 weeks [49.20±13.28]% and (75.94±8.89)% respectively] and 12 weeks [(52.08±16.78)% and (71.8±13.17)% respectively, Fig. 8]. Only group-3 showed significant increase (2.5 fold) in development of mature blood vessels [from (35.4±9.68)% at 6 weeks to (90.6±19.54)% at 12 weeks, P<0.001]. The mature blood vessel index in the group-4 was significantly higher than any other group at 6 weeks after treatment (P<0.05). At 12 weeks after treatment, mature blood vessel density of the groups-3 and -4 was significantly higher than any other group (P<0.005).

The fluorescent microsphere study showed a significant improvement of regional blood flow in groups-3 and -4. In the center of the infarction, the regional blood flow (ml/min/g) of groups-3 and -4 continuously increased from 0.55±0.13 and 0.61±0.03 at 0 week after cell transplantation (3 weeks after model establishment) to 1.29±0.16 and 1.51±0.17 at 6 weeks, 1.73±0.19 and 2.14±0.13 at 12 weeks after cell transplantation (Fig. 9a). These values were significantly higher than those of the groups-1 and -2 at 6 weeks (0.66±0.16 and 0.81±0.16) (P<0.001) and 12 weeks (0.39±0.13 and 1.0±0.14) (P<0.001).

At the peri-infarct region, the blood flow of the groups-3 and -4 continuously increased from 0.93±0.17 and 0.97±0.06 at 0 weeks after cell transplantation to 2.41±0.28 and 2.73±0.07 at 6 weeks, 3.39±0.24 and 3.59±0.46 at 12 weeks after cell transplantation (Fig. 9b). They were significantly higher than
the groups-1 and -2 at 6 weeks (1.22±0.45 and 1.14±0.09, *P*<0.001) and 12 weeks after cell transplantation (0.98±0.10 and 1.38±0.25, *P*<0.001).

**Discussion**

The study compared the effects of hVEGF$_{165}$ and Ang-1 overexpression after SKM based delivery of their respective gene, on neovascularization in a porcine model of chronic myocardial infarction. The results demonstrated functionally improved neovascularization with increased blood flow after transplantation of SKM carrying hVEGF$_{165}$ or Ang-1. Current study also highlights that Ang-1 may be a more potent arteriogenic inducer for neovascularization.

Cell mediated angiogenic gene transfer is a superior approach over conventional therapeutic angiogenesis. It has been demonstrated that SKM are excellent carriers for transgene delivery to achieve cellular angiogenesis. The cell based therapeutic gene delivery approach is advantageous, and allows localized and regulatable expression of the transgene together with achieving concurrent angiomyogenesis. In the early phase after cell transplantation, the angiogenic proteins expressed at the site of the graft may provide a cytoprotective and cardio-protective effect. On the longer-term basis, induction of neovascularization at the site of cell graft in the infarcted myocardium leads to improved regional blood flow and hence alleviation of tissue ischemia.

We had reported that SKM transected with adenoviral vector carrying either VEGF$_{165}$ or simultaneously VEGF$_{165}$+Ang-1 was able to transiently express angiogenic proteins up to 30 days at the site of the cell graft. Suzuki and colleagues have investigated the efficacy of hVEGF$_{165}$ transfected rat primary SKM transplantation for the improvement of injured heart. Myocardial VEGF levels in the experimental animals increased significantly between day 2 and day 14 after transplantation with enhanced angiogenesis without angioma formation. The transplantation of genetically modified SKM showed superiority over non-transduced SKM transplantation in improvement of heart function.

We have investigated the functional angiogenic response to hVEGF$_{165}$ or Ang-1 delivery in short (6 weeks) and a long (12 weeks) terms during the cell graft. Extensive capillary formation was observed at 6 weeks (vWF-VIII= 57.13±4.19) in the group-3. However, the blood vessel density based on vWF-VIII immunostaining significantly declined to 32.1±1.74 at 12 weeks. The possible explanation for this observation was that the over expression of hVEGF$_{165}$ resulted in the development of blood vessel conduits constituted by endothelial cell aggregates. These blood vessel conduits lacked smooth muscle covering as indicated by the low maturation index (35.4%) in group-3. With lack of maturation signals, at 12 weeks, most of these endothelial aggregates regressed thus lowering down the blood vessel density. Different from VEGF$_{165}$ a primary initiator of new capillary growth, Ang-1 is mainly involved in late stage to stabilize newly formed capillary. Current study demonstrated that Ang-1 not only accelerated blood vessel maturation process, but also increased capillary blood vessel density (vWF-VIII= 39.9±3.09) at 6 weeks. This was consistent with studies of Shyu et al., and Gurunluoglu et al. At 6 weeks, the blood vessel density in group-4 was lower than that of group-3. However, it was higher as compared with groups-1 and -2 suggesting that the Ang-1 delivered by SKM also stimulated capillary formation. Although it lacks mitotic activity on endothelial cells, Ang-1 can induce blood vessel formation in vivo by stimulating migration and sprouting of endothelial cells. It is also reasonable that Ang-1 induced fewer capillaries than VEGF$_{165}$ which had pro-mitotic activity on endothelial cells. Dual fluorescent immunostaining for vWF-VIII and SMA expression further demonstrated that (85.2±2.86)% of these blood vessels were coated by smooth muscle cells at 6 weeks. This is consistent with the biological activity of Ang-1 which mainly involved remodeling of the newly formed capillaries. At 12 weeks, as most of the blood vessels stimulated by Ang-1 were having smooth muscle cell covering and became stably mature vessels and hence an increased blood vessel maturation index was achieved in group-4.

The ultimate aim of neovascularization is to reconstruct blood vessel network that can normalize or improve perfusion in ischemic myocardial. Microsphere study demonstrated maximally increased regional reperfusion was achieved in group-4 followed by group-3. Both the groups had significantly increased regional reperfusion in the center and peri-infarct area as compared with groups-1 and -2 at 6 and 12 weeks. These results demonstrate that neovascularization achieved after Ang-1 overexpression resulted in the development of functionally competent blood vessel formation.

Based on these data, we concluded that transient overexpression of hVEGF$_{165}$ or Ang-1 from SKM could be achieved by transduction with recombinant adenoviral vectors encoding for the respective genes. The SKM with overexpressed angiogenic gene served as a reservoir of angiogenic transgenes at the site of the cell graft in infarcted heart. The hVEGF$_{165}$ delivered by SKM could efficiently induce capillary formation, while Ang-1 induced arteriole formation. Although over expression of both VEGF$_{165}$ and Ang-1 efficiently induced functionally competent blood vessel formation, the angiogenic effects of Ang-1 overexpression resulted in mature and stable blood vessel formation.

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References


