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High efficiency transduction of human $VEGF_{165}$ into human skeletal myoblasts: *in vitro* studies

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Abbreviations: HM, Human myoblasts; HUVEC, Human umbilical vein endothelial cells; $hVEGF_{165}$, Human $VEGF_{165}$; $hVEGF_{165}$-HM, Human $VEGF_{165}$ transduced human myoblast; Null Ad-HM, Null adenoviral vector transduced human myoblast; RT, reverse transcription

Abstract

We report the transduction of human $VEGF_{165}$ gene into human myoblast and characterization of the transduced myoblasts for transduction and expression efficiency. Human myoblasts were assessed by immunostaining for desmin expression. A replication incompetent adenoviral vector carrying human $VEGF_{165}$ was constructed and used for transduction of myoblasts. Immunostaining of transduced myoblasts was used to determine transduction efficiency. Expression efficiency was confirmed by immunoblotting, ELISA and reverse transcription (RT)-PCR analysis using human-$VEGF_{165}$ specific primers (5'-3' = 5'ATGAACTTTCTGCTGTCTTGGGTG and 3'-5' = ACACCGCCTCGGCTTGTCACA3'. Biological activity of the secreted $VEGF_{165}$ was determined by human umbilical vein endothelial cell proliferation and [H^3] thymidine incorporation assays. Human myoblast preparation was >95% pure with 99% viability after transduction. Immunostaining showed >95% $VEGF_{165}$ positive myoblasts. Western blotting and ELISA revealed high $VEGF_{165}$ expression in the transduced myoblasts. Maximum transduction efficiency was achieved by 8 h exposure of myoblasts to virus at 1:1,000 ratio on three consecutive days. Concentration of $VEGF_{165}$ released in the culture medium peaked (37±3 ng/ml) at 8 days post-transduction. Cell proliferation assay on human umbilical vein endothelial cells using supernatant from $VEGF_{165}$ transduced myoblasts revealed extensive proliferation of cells which was suppressed in the presence of anti-human $VEGF_{165}$ antibody in culture medium and was further confirmed by thymidine incorporation assay. The untransduced myoblasts secreted $VEGF_{165}$ *in vitro* (300± 50 pg/ml) that is enhanced many folds (37±3 ng/ml) in $VEGF_{165}$ transduced myoblast as determined by ELISA. These studies suggest that human myoblast are potential carriers of human $VEGF_{165}$ to achieve concurrent angiomyogenesis for cardiac repair.

Keywords: adenovirus; angiogenic factor; gene therapy; genetic vectors; myoblasts; neovascularization

Introduction

Despite advances in the surgical and therapeutic revascularization techniques, treatment of ischemic heart disease and peripheral vascular disease remains a challenge. Gene transduction for therapeutic angiogenesis is being considered as a potential alternative strategy (Abo-Auda *et al*., 2003; Merkle *et al*., 2003). Myocardial ischemia strongly triggers the release of various angiogenic factors and chemokines (Helisch *et al*., 1999). More than 20 molecules with angiogenic potential including acidic and basic fibroblast growth factors (aFGF and bFGF), angiopoietins (Ang) including Ang-1 and Ang-2, VEGF isoforms, hepatocyte growth factor (HGF), chemokines including IL-1, IL-8 and MCP-1 have been identified and assessed for their role in angiogenesis (Koblizek *et al*., 1998; Gonacalves *et al*., 2000; Jain *et al*., 2000; Poltorak *et al*., 2000; Ahmet *et al*., 2003). The more well-documented are the VEGF isoforms due to their pivotal role in angiogenesis (Lee *et al*., 2002).

Angiogenesis being a cascade of sequential events needs a coordinated and concerted involvement of various types of cells under the influence of different mitogens (Carmiliet 2000). Two commonly adopted

approaches for therapeutic angiogenesis include protein therapy using direct injection of recombinant cytokines and the gene therapy approach based on the delivery of gene encoding for one or more angiogenic factors (Dazu *et al* 2001; Khan *et al*., 2003). The later strategy has been accomplished using naked DNA injection, non-viral vectors and viral vectors into the myocardium (Rosengart *et al*., 1999; Kornowski *et al*., 2000; Kay *et al*., 2001; Tse *et al*., 2003). A more recent diversification from these two basic strategies is to achieve angiogenesis through transplantation of cells (Chekanov *et al*., 2003; Nishida *et al*., 2003; Stamm *et al*., 2003).

The feasibility of ex-vivo cell mediated gene transfer is being widely investigated (Pagel *et al*., 1995; Floyd *et al*., 1998). More recently, genetically engineered myoblast mediated FGF2 delivery for revasularization in a model of acute skin flap ischemia has been documented (Rinch *et al*., 2001). Similar reports have also been published for angiogenic gene delivery to the myocardium using myoblast (Suzuki *et al*., 2001; Yau *et al*., 2001). We have already reported human myoblasts (HM) carrying β-galactosidase reporter gene as a model for concomitant cell transplantation and therapeutic gene delivery to the skeletal muscle as well the myocardium in the animal models (Haider *et al*., 2002). The transplanted HM were found to survive and express the reporter gene up to 30 weeks after transplantation. We are reporting a highly efficient, adenoviral vector mediated hVEGF$_{165}$ gene transduction into HM and their characterization for transduction and expression efficiency. We reckon that the use of HM as carriers of exogenous human angiogenic gene will be safer for future *in vivo* studies.

Materials and Methods

Cell culture

Human myoblasts were manufactured according to the in-house Standard Operating Protocols (SOP) and trade secrets with a license of the U.S. Patent No. 5,130,141 (Law *et al*., 1992). They were cultured in Cell Transplants Singapore Pte. Ltd., according to current Good Manufacturing Practice (cGMP) and ISO9001. At harvest, the yield was $>$98% pure for human myoblasts. The cells were maintained with patented Super Medium (Cell Transplantation Inc., Singapore) containing 10% FBS at 37^{o}C in 5% CO_2 incubator until confluent and were frequently passaged after every 48-72 h. HEK-293 cells (human embryonic kidney cells line) were obtained from Dr. Ge Rouwen, Biological Sciences Lab, National University of Singapore and maintained in DMEM. Human umbilical vein endothelial cell (HUVEC) was gifted by Dr. Song Jie, Atherosclerosis Research Lab, National University of Singapore. The cells were grown in F-12K medium (ATCC). HeLa cells were a gift from Associate Prof. Hanry Yu, Department of Physiology, National University of Singapore. All the cells lines were maintained in their respective basal medium supplemented with 1% penicillin/steptomycin, 2% glutamine and 10% FBS. The cells were cultured at 37^{o}C in 5% CO_2.

Construction and purification of adenovirus vector carrying human VEGF$_{165}$

The recombinant adenovirus was packaged using HEK-293 cells. The adenoviral shuttle plasmid pCA14 carrying human VEGF$_{165}$ (hVEGF$_{165}$) driven by immediate early human cytomegalovirus promoter was rescued into the deleted E1 region of the pJM17 Ad5 genomic plasmid by co-transfection into HEK-293 cells. The virus was plaque purified thrice before amplification. Cell density of about 80-90% confluence was infected in 2% FBS supplemented DMEM. Following full cytopathic effect development, cells were harvested, repeated freeze/thaw and purified by cesium chloride gradient ultracentrifugation following standard procedures. The viral titer was estimated by endpointassay (Quantum Biotechnology) and the virus particle was calculated based on 1 optical density equivalent to 1.25×10^{12} particles/ml. The recombinant virus was confirmed replication-incompetent with undetectable E1 DNA by PCR.

Adenovirus carrying human VEGF$_{165}$ (Ad-hVEGF$_{165}$) was propagated in HEK-293 cells, cultured at a cell density of 1×10^{6} cells in 75-mm^2 tissue culture flasks using DMEM cell culture medium supplemented with 10% FBS. At 70% confluence, the cells were infected with Ad-hVEGF$_{165}$ or Null-Ad (Null adenovirus). At stipulated time, the supernatant from 293 cells was removed, centrifuged to remove any cell debris and used for transduction of HM.

Immunostaining for desmin expression

Human myoblast was cultured on poly-lysine coated microscopic glass slides. The cells were immunostained using desmin immunstaining kit as per manufacturer's instructions (Sigma). The proportion of desmin positive myoblasts was calculated from the ratio between stained and unstained cell counting the various microscopic fields.

Transduction of human myoblasts with hVEGF$_{165}$

Human myoblasts was cultured at 1×10^{7} cell density in 225-mm^2 tissue culture flasks. The cells were exposed to various virus: cell number ratios for 2 h, 4 h, 8 h, and 24 h. At pre-determined time intervals

post-infection, the virus infection medium was replaced with normal super medium for 24 h. The transduction procedure was repeated three times to optimize transduction conditions.

Characterization of $hVEGF_{165}$ transduced human myoblasts

The detection of $hVEGF_{165}$ secreted from the transduced HM was carried out using $hVEGF_{165}$ Sandwich ELISA kit (Chemicon International Inc., US). The human $hVEGF_{165}$ transduced human myoblasts ($hVEGF_{165}$-HM) were grown in six-well tissue culture plates at a cell density of 2×10^5 cells/well, using human myobalsts transduced with null adenovirus vector (Null Ad-HM) and non-transduced myoblasts as control. The supernatant from each well was collected at regular time intervals from day 1 for up to 18 days at two days interval and kept frozen at -20°C until used for assay. The assay was performed as per instructions of the supplier. Briefly, 100 l samples or $hVEGF_{165}$ standards were coated into each designated well in triplicate and 25 l of diluted biotinylated rabbit anti-$hVEGF_{165}$ polyclonal antibody was dispensed into each well. The plate was incubated at room temperature for 3 h, washed three times with wash buffer and incubated at room temperature for 45 min with streptavidin conjugated alkaline phosphatase. The presence of primary antibody was detected by color reagent system and absorbance was determined at 490 nm using ELISA plate reader (SLT Lab Instruments, Australia).

Western blot analysis

The HM were grown in six well tissue culture plates and transduced with Ad-$hVEGF_{165}$ or null adeno-virus as described above. Samples from each well were obtained at 48 h after transduction. Cell samples were washed three times with PBS. The cells were resuspended in 0.5 ml PBS and lysed by 5 cycles of freeze and thaw. The cell extract was centrifuged at 5,000 rpm to remove cell debris. The supernatant was used as the cell lysate for immunoblotting. The aliquots of the samples were boiled in denaturing sample buffer and separated on 10% SDS polyacrylamide gel under reducing conditions using 50 mM Tris/glycine buffer pH 8.8. The separated proteins were transferred onto nitrocellulose membrane (Sigma Chemicals) for Western blot analysis and processed for the detection of $hVEGF_{165}$. The blots were washed three times with 10 mM Tris/HCl wash buffer pH 7.6 containing 0.05% Tween-20. The membrane was incubated at room temperature for one hour in 2% bovine serum albumin in wash buffer to prevent any non-specific antibody binding. The membrane was then incubated with 1:500 dilution of anti-$hVEGF_{165}$ specific polyclonal antibody (Chemicon). The antibody binding was visualized with diaminobenzidine visualization system.

Endothelial cell proliferation assay

HUVEC cells were cultured in F-12K medium supplemented with 10% fetal bovine serum and 20 U/ml heparin. For cell proliferation assay, 1×10^5 cells/well were cultured in six well tissue culture plates in triplicate for each sample. After 24 h culture with DMEM containing 2% FBS, the cells were washed twice with PBS and medium from Ad-$VEGF_{165}$-HM, non-transduced, Null Ad-HM, simple growth medium and Ad-$hVEGF_{165}$ medium with 1:1,000 anti-$hVEGF_{165}$ antibody. The cells were grown for 72-96 h at 37°C in 5% CO_2, harvested by trypsinization using 0.1% trypan blue and counted.

[H^3] thymidine incorporation assay

HUVEC were cultured in F-12K medium supplemented with 10% FBS and 20 units/ml heparin. For thymidine [H^3] incorporation assay, 1×10^5 cell/well were seeded in 6-well tissue culture plates in triplicate for each sample. After 24 h culture with DMEM containing 2% FBS, the cells were washed twice with PBS and medium from Ad-$hVEGF_{165}$-HM, non-transduced, null Ad-HM, super medium and Ad-$hVEGF_{165}$-HM medium with 1:1,000 anti-$hVEGF_{165}$ antibody. Each well was supplemented with 2 Ci/ml [H^3] thymidine (ICN Biomedical Inc.). The cells were harvested after 72-96 h and thoroughly washed with ice- cold PBS to remove unincorporated [H^3] thymidine. The cells were lysed using 0.5 ml of 10.25 N NaOH and centrifuged at 5,000 rpm for 10 min to remove the cell debris. The clear supernatant (400 l) was mixed with 4 ml scintillation buffer (Ready Safe, Beckman) and radioactivity counts were measured using Beckman LT-6500 Multipurpose Scintillation Counter (USA).

Immunohistochemical staining

Ad-$hVEGF_{165}$-HM and null Ad-HM vector were grown on poly-lysine coated glass microscopic slides for 48 h. After washing with PBS containing 0.05% Tween-20 as wash buffer, the cells were fixed with -20°C cold methanol for 10 min. After blocking for one hour with 1% BSA in the wash buffer to avoid non-specific antibody reaction, the cells were incubated at 37°C with 1:500 dilution of anti-$VEGF_{165}$ primary antibody (Chemicon). One hour later, the cells were washed three times with wash buffer. The secondary antibody-HRP enzyme conjugate (1:200) was added for one hour at room temperature. Finally, the cells were washed three times and visualized with DAB visualization system.

Reverse transcription (RT)-PCR analysis for hVEGF$_{165}$ gene expression in transduced human myoblast

Samples from pure HM, null Ad-HM, Ad-hVEGF$_{165}$-HM (on days 1, 8, and 18 days) were used for RT-PCR analysis. The RT-PCR primers of hVEGF$_{165}$ 5'→3' (5' ATGAACTTTCTGCTGTCTTGGGTG 3') and 3'→5' (5' TCACCGCCTCGGCTTGTCACA 3') and GAPDH 5'→ 3' (5' GTGGAGGAGTGGGTGTCGCTG 3') and 3'→5' (5' CGGATTTGGTCGTATTGGGCG 3') were used for amplification. The T_m and annealing temperatures for VEGF$_{165}$ were 68-70°C and 64°C and for GAPDH were 66-70°C and 64°C respectively.

The total RNA is isolated by using total RNA Isolation Kit (Purescript). Briefly, 300 l cell lysis solution is added into each sample for 3 min after cells are collected. The 100 l protein-DNA precipitation solution is added to the cell lysate for 5 min and centrifuge. The supernatant was collected and mixed with 300 l 100% isopropanol. RNA was recovered by centrifugation followed by the addition of 50 l RNA hydration solution to rehydrate isolated RNA. The purified RNA was kept at -80°C until use.

The RT-PCR of HM was done by using One-step RT-PCR Kit (Qiagen). Briefly, 10 master mixtures were prepared of 10 samples (five with VEGF$_{165}$ primers, five with GAPDH primers). 500 ng template total RNA of each sample was added into each master mixtures. The reverse transcription was carried out at 50°C for 30 min followed by initial PCR activation step for 15 min at 95°C. The denaturation and annealing temperatures were 94°C and 64°C respectively. The reaction was carried out for a total of 30 cycles, followed by final extension at 72°C for 10 min.

Results

The HM culture was more than 98% pure population of the cells as assessed by desmin staining (Figure 1). Transduction efficiency revealed a vector dose dependent relation between the viral particle number and HM (Figure 2). Similarly, the time of exposure of HM to Ad-VEGF$_{165}$ showed a direct relation with transduction efficiency (Figure 3). However, at a longer time exposure and at a higher viral titer, the HM showed ill effects towards viral exposure. Repeated transductions of HM improved transduction efficiency. Optimum level of transduction efficiency was achieved at 1:1,000 HM: viral ratio when transduction was carried out for 8 h exposure, three times at an interval of 24 h after every transduction (Figure 2 and 3). The cell viability was > 99% by dye exclusion method using Trypan Blue staining after transduction. ELISA results revealed that the transduced HM continued to secrete hVEGF$_{165}$ for up to 30 days of observation after transduction, day '0', 24 h (5±1 ng/ml), 2 days (8±2 ng/ml) reaching peak (37±3 ng/ml) of VEGF$_{165}$ in the cell culture supernatant at 7 days (Figure 4). Our results also showed that the untransduced HM or Null Ad-HM also secreted hVEGF$_{165}$, however, the level of secretion was very low (300±50 pg/ml, 800± 150 pg/ml respectively). These findings have been confirmed by immunoblotting and RT-PCR results. Immunoblotting on HM culture supernatant and cell lysate, using anti-hVEGF$_{165}$ showed the presence of hVEGF$_{165}$ in the medium and cell lysate. This showed that hVEGF$_{165}$ is actively secreted in the medium of hVEGF$_{165}$-HM. Immunochemical staining of hVEGF$_{165}$-HM revealed high transduction efficiency of more than 95% for hVEGF$_{165}$ (Figure 5). Our RT-PCR results

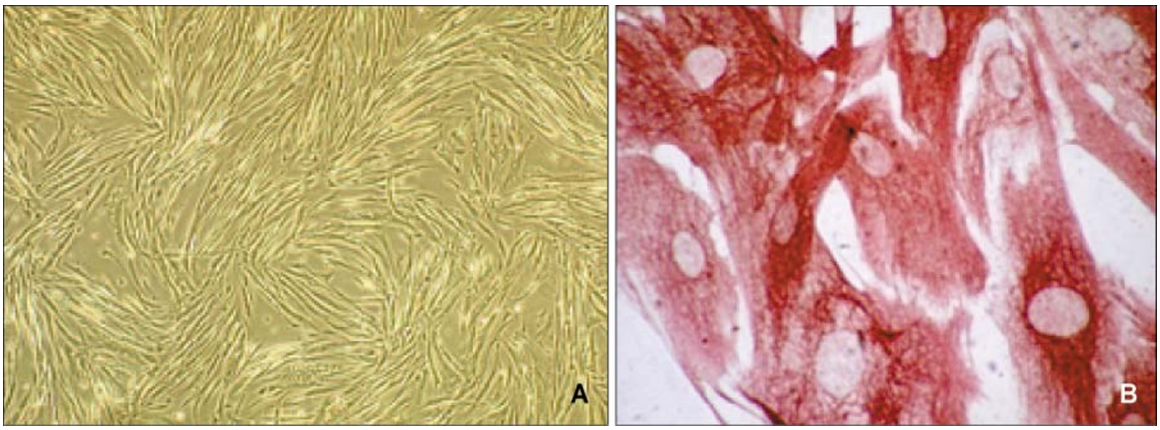

Figure 1. (A) Phase contrast photomicrograph of HM culture. The cells were propagated in poly-lysine coated tissue culture flasks using patented Super Medium. (B) Immunostaining of HM for the expression of desmin.

comply with the findings of ELISA and immunoblotting. The presence of mRNA encoding for $hVEGF_{165}$ as detected by $VEFG_{165}$ specific primers revealed high levels of $VEGF_{165}$ gene expression (Figure 6).

The biological activity of $hVEGF_{165}$ secreted from transduced HM was assessed through HUVEC proliferation and Thymidine [H^3] incorporation assays. VEGF promotes endothelial cell proliferation and triggers DNA synthesis by the endothelial cells. The two control groups of un-transduced HM and Null Ad-HM showed poor rate of proliferation as compared with experimental group (Figure 7A and B). The effect of $hVEGF_{165}$ triggered proliferation of HUVEC was inhibited by $hVEGF_{165}$ specific polyclonal anti-sera (Figure 7A and B). This is further supported by the results from thymidine incorporation assay which confirmed the biological activity of the HM secreted $hVEGF_{165}$ (Figure 8).

Discussion

The use of HM for $hVEGF_{165}$ transduction in our study plan is to establish experimental conditions for angio-

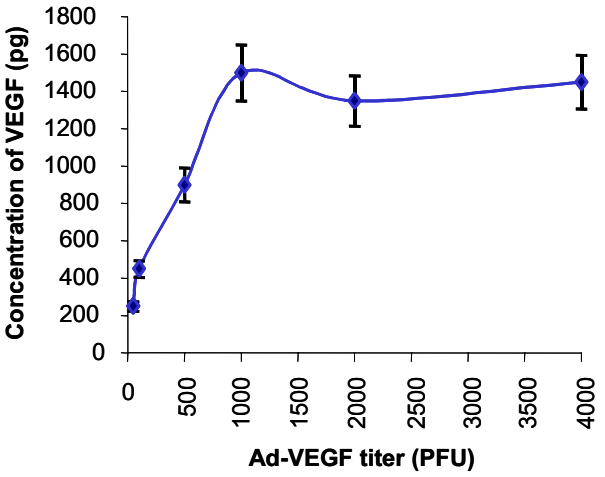

Figure 2. Transduction efficiency as a function of the ratio between Ad-$hVEGF_{165}$ and HM. The transduction efficiency was maximum at 1,000 PFU: 1 HM.

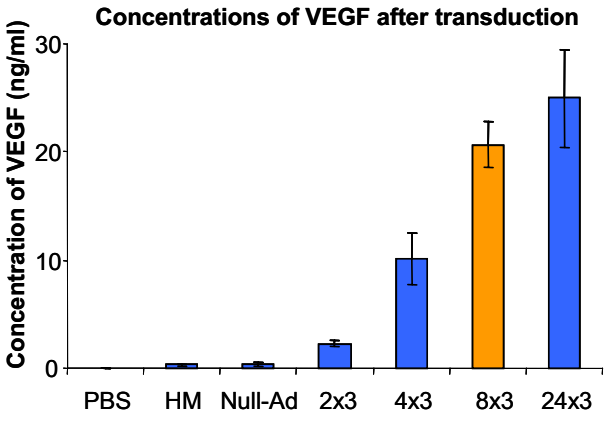

Figure 3. Expression of $hVEGF_{165}$ from Ad-$hVEGF_{165}$-HM as a function of transduction time. With an increase in transduction time, the level of $hVEGF_{165}$ expression increased accordingly. Although the three times transduction, each for 24 h, gave highest transduction efficiency, a substantial number of HM died. The three times transduction, 8 h each time, was well tolerated by HM.

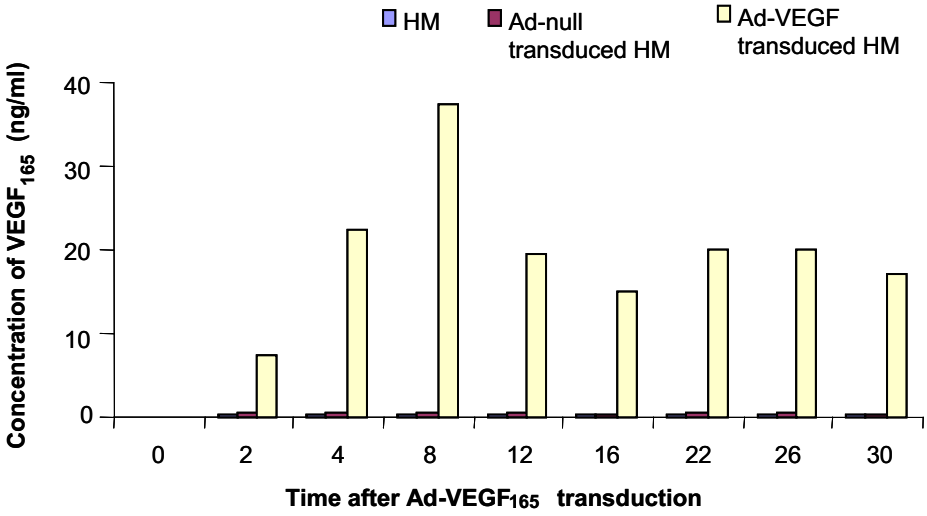

Figure 4. Expression of $hVEGF_{165}$ protein from Ad-$hVEGF_{165}$-HM as a function of time. The peak of secreted $hVEGF_{165}$ (37±3 ng/ml) was at 7 days post transduction, declining to 19±2 ng/ml at 30 days post transduction. Untransduced HM also secreted $hVEGF_{165}$ at a low level (0.3±0.05 ng/ml) as compared to the null Ad-HM (0.8±0.1 ng/ml).

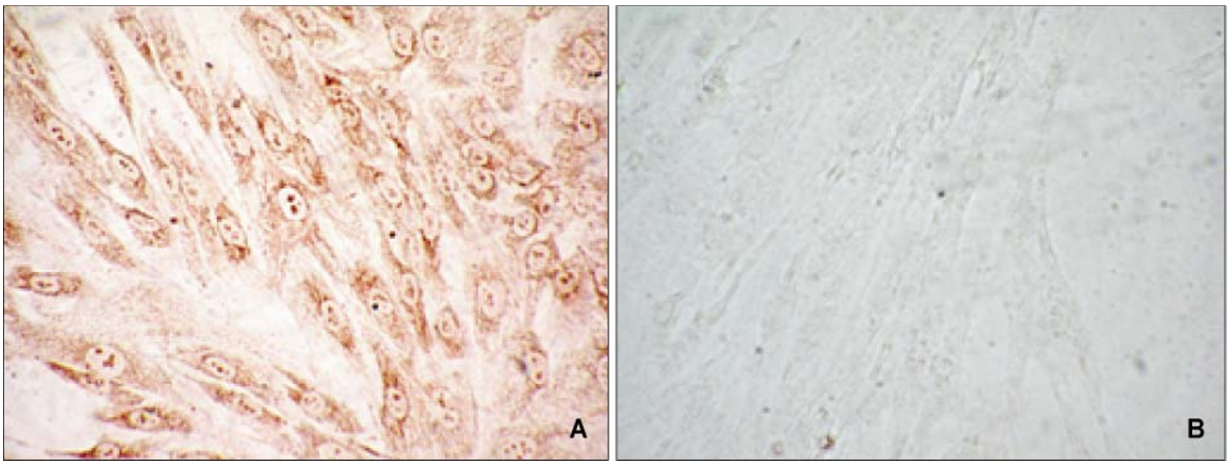

Figure 5. Immunostaining of Ad-hVEGF$_{165}$ transduced HM for hVEGF$_{165}$ expression. (A) Ad-VEGF$_{165}$-HM expressing VEGF$_{165}$ (B) Null Ad-HM.

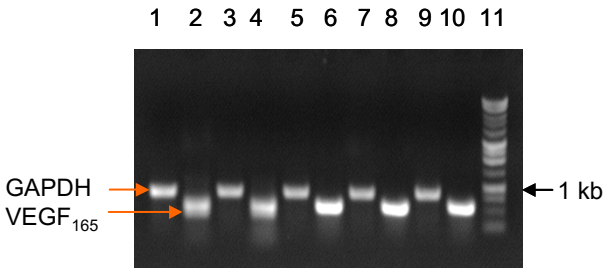

Figure 6. RT-PCR analysis of Ad-hVEGF$_{165}$ transduced HM using human hVEGF$_{165}$ specific primers. The hVEGF$_{165}$ mRNA of Ad-VEGF$_{165}$ transduced HM was much stronger than pure HM and Null Ad-HM, even at 18 days after transduction. Samples 1&2 = HM, Samples 3&4 = null Ad-HM, Samples 5-10 = hVEGF$_{165}$-HM at 1, 8, and 18 days post transduction, Sample 11 = DNA ladder.

myogenesis using autologous HM for hVEGF$_{165}$ gene delivery. Successful gene transfer with demonstration of high efficiency transfection and high level of transgene expression in diseased myocardium has been reported (Hertulla *et al*., 2000).

VEGF$_{165}$ gene has been transferred into the myocardium as a naked plasmid or as a part of viral vector cassette in animal models and human studies (Lopez *et al*., 1998; Vale *et al*., 2000). Attempts have also been made for site-specific catheter based delivery of angiogenic vectors for enhanced neovascularization (Laitinen *et al*., 2000). Apart from the conventional methods, implantation of genetically engineered cells is gaining popularity these days due to its promise in tissue engineering to repair damaged myocardium secondary to myocardial infarction (Springer *et al*., 2000). One of the more fascinating approaches in the field of cardiovascular gene therapy would be to achieve therapeutic angiogenesis to enhance collateral development and tissue perfusion in the ischemic area alongside cellular myocardial reconstruction to strengthen the weakened heart muscle in the scar tissue. The potential advantage of this technique is to create a reservoir of myogenic cells that would establish as a part of the myocardial tissue. These transplanted cells will provide a transient source of angiogenic factors such as VEGF following transplantation. This may help to maintain a therapeutic level of these factors for angiogenesis in combination with tissue repair.

Most of the research on angiogenesis is focused on the use of VEGF, which is a 34-36-kDa heparin binding glycoprotein having specific receptors on the endothelial cells (Ferrara, 1999). VEGF isoforms, VEGF$_{165}$ and VEGF$_{121}$ are capable of efficient stimulation of angiogenesis and are being widely considered for research and clinical application (Merkle *et al*., 2003). The use of HM for cardiac muscle grafting with an added intention of transgene transfer is a two pronged strategy. The transplanted myoblasts strengthens the infarcted tissue on one hand and simultaneously serve as carriers of therapeutic gene for angiogenesis. The neovascularization thus produced enhances the probability of grafted cell survival in the otherwise hostile ischemic tissue environment.

In our present study, we have successfully transduced HM using Ad-hVEFG$_{165}$ vector. The transduction efficiency and the release of the expression product of the exogenous transduced gene were very high (37±3 ng/ml). The hVEGF$_{165}$ secreted from the transduced cells was identified as a 42 kDa molecular weight protein as determined by immunoblotting. The results of HUVEC proliferation assay (Figure 7A and B) and Thymidine incorporation assay (Figure 8) showed that the hVEGF$_{165}$ secreted from HM was biologically active. It triggered DNA synthesis and proliferation of endothelial cells. Furthermore, the

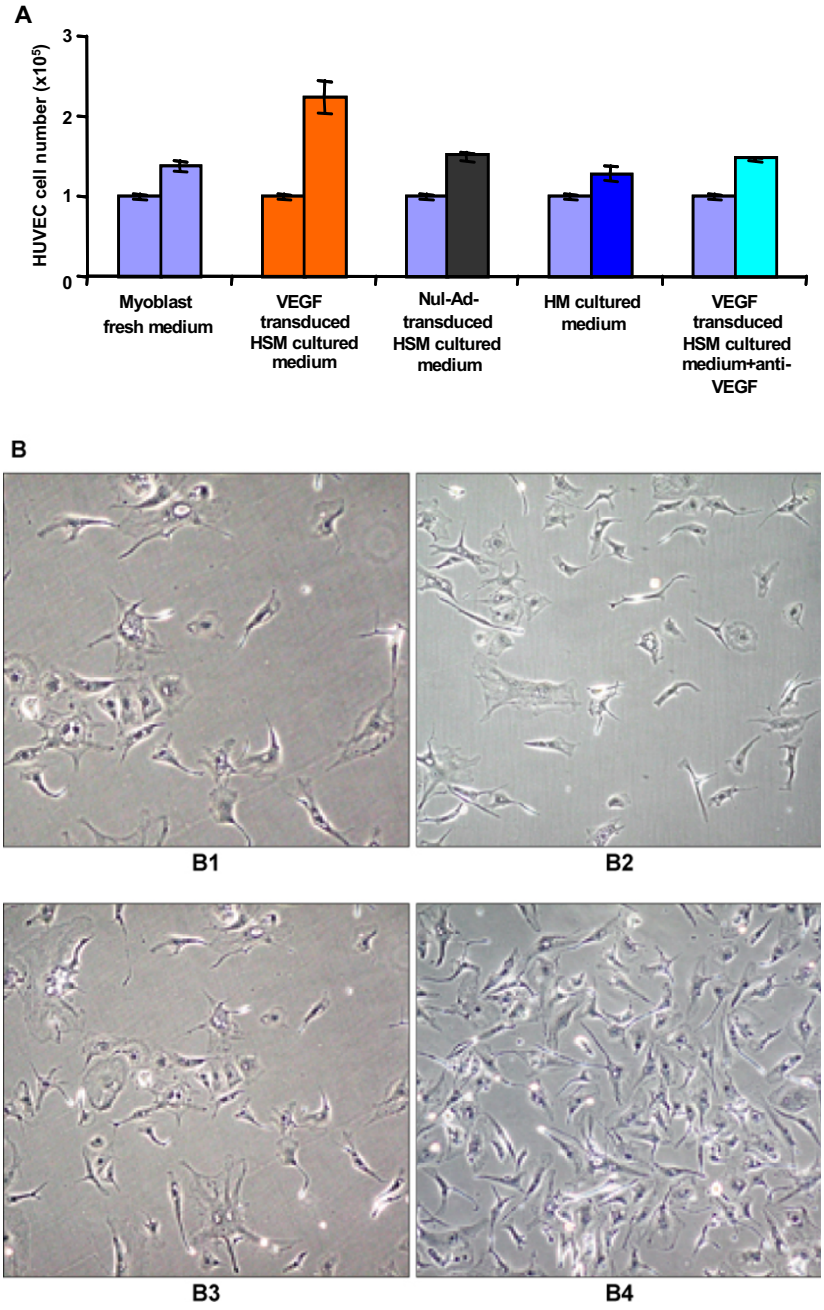

Figure 7. A, Cell proliferation assay using HUVEC to assess the biological activity of $hVEGF_{165}$ secreted from Ad-$hVEGF_{165}$-HM. B, Conditioned medium were used to assess the proliferation of HUVEC. B1, untransduced HM; B2, Null Ad-transduced HM; B3, $hVEGF_{165}$-HM supernatant pre-treated with anti-$VEGF_{165}$; B4, Ad-$hVEGF_{165}$-HM.

transduced HM persistently secreted $VEGF_{165}$ for up to 30 days of observation with peak levels reaching at about 7 days after gene incorporation experiment (Figure 4 and 6). These results were in harmony with some of the previously published data (Floyd *et al.*, 1998).

One critical aspect in *ex-vivo* gene delivery is the duration of transgene expression after cell transplantation. Depending upon the mode of gene transfer into myoblasts, the gene expression has been reported to persist from 5 weeks to 3 months to 7 months, for up to a year (Lee *et al.*, 2000; Rinsch *et al.* 2001; Suzuki *et al.*, 2001; Yau *et al.*, 2001). High transduction efficiency and transient expression are the important features of adenovirus-mediated transduction (Benihoud *et al.*, 1999). We hypothesized that a transient expression of the angiogenic gene would be sufficient to initiate the process of neovascularization. The adenoviral vector construct for VEGF delivery to the myoblasts during the present study was intended to have transient expression of VEGF to initiate angiogenesis. We hypothesize that a longer term ex-

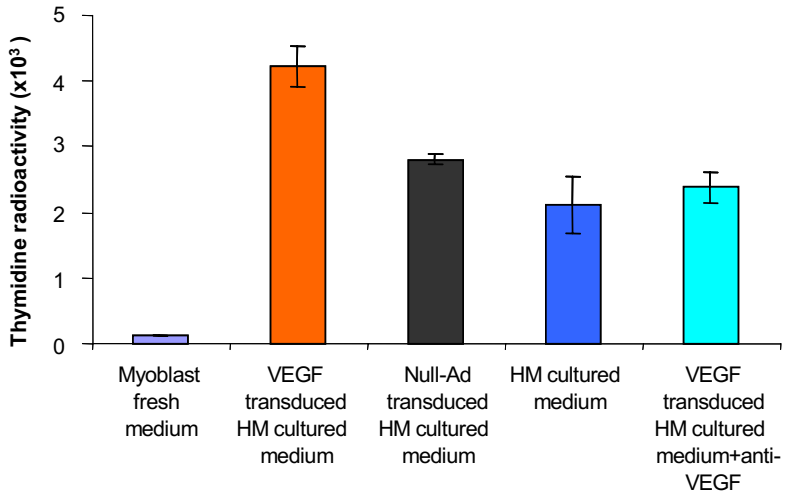

Figure 8. Thymidine incorporation assay using HUVEC as a model cell line to assess the biological activity of hVEGF$_{165}$ secreted from Ad-hVEGF$_{165}$-HM. Cell culture supernatants from Null Ad-HM, untransduced HM and hVEGF$_{165}$-HM supernatant pre-treated with anti-VEGF$_{165}$ antibody and Ad-hVEGF$_{165}$-HM were used to assess the [^{3}H] thymidine (2 Ci/well) uptake by HUVEC, fresh Super Medium as a negative control.

pression is not needed and may lead to deleterious effects.

Once the process has set in, further expression of exogenous gene is undesired and may lead to untoward pathological events (Lee *et al*., 2000). In addition to this, it has been reported that direct injection of adenoviruses carrying exogenous gene may give rise to inflammatory reactions and immune response from the host defense system (Kay *et al*., 2001). The cell-based delivery is a safe alternative for angiogenic gene delivery to the myocardium. As our findings suggested that autologous donor cells continued to secrete VEGF$_{165}$ for 30 days, time duration long enough to initiate and set in progression the course of neovascularization (Figure 4). As observed during the current study, transduction with Ad-VEGF$_{165}$ had no bad effects on HM in the tissue culture, the cell proliferation continued unabated and cell viability post-transduction remained $>$ 99%. Hence, it was expected that these cells upon transplantation would go on to differentiate and form muscle fibers in the host myocardium for cellular cardiomyoplasty.

To conclude, we have demonstrated hVEGF$_{165}$ transduction into HM for concurrent application of cell transplantation and angiogenesis. The use of autologous cells for transplantation and human angiogenic factor for neovascularization will be much more effective and less immunogenic.

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